

Preparation of colloidal particles combining conducting polymers with biopolymers

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Master's Thesis
2025



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Akademický rok: 2024/2025

ZADÁNÍ DIPLOMOVÉ PRÁCE

(projektu, uměleckého díla, uměleckého výkonu)

Jméno a příjmení: Bc. Markéta Vachůnová
Osobní číslo: T23249
Studijní program: N0711A130011 Biomateriály a kosmetika
Forma studia: Prezenční
Téma práce: Příprava koloidních částic kombinujících vodivé polymery s biopolymery

Zásady pro vypracování

Studentka zpracuje rešeršní část práce zahrnující definování vlastností vodivých polymerů a biopolymerů klíčových pro jejich využití v biomedicině.

Soustředí se především na polypyrrol a chitosan.

Dále stručně popíše postupy přípravy koloidních systémů.

V praktické studentka částí připraví koloidní částice založené na kombinaci vodivých polymerů a biopolymerů a následně provede jejich biologickou a materiálovou charakterizaci.

Forma zpracování diplomové práce: **tištěná/elektronická**
Jazyk zpracování: **Angličtina**

Seznam doporučené literatury:

- [1] Synthesis of Conductive Polymer-based Composite Particles; CHEMISTRY LETTERS; DOI 10.1246/cl.230235
- [2] Nano-and Microemulsions in Biomedicine: From Theory to Practice; PHARMACEUTICS; DOI 10.3390/pharmaceutics15071989
- [3] Tailoring of conducting polymers via copolymerization-A review; EUROPEAN POLYMER JOURNAL; DOI 10.1016/j.eurpolymj.2021.110561
- [4] Conductive polymers: Towards a smart biomaterial for tissue engineering; ACTA BIOMATERIALIA; DOI 10.1016/j.actbio.2014.02.015

Vedoucí diplomové práce: **prof. Ing. Petr Humpolíček, Ph.D.**
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Datum zadání diplomové práce: **10. února 2025**
Termín odevzdání diplomové práce: **9. května 2025**

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ABSTRAKT

Tato diplomová práce se zaměřuje na syntézu, charakterizaci a biologické hodnocení koloidních částic složených z polypyrrolu a chitooligosacharidů s různou délkou řetězce. Částice byly připraveny bez použití oxidačního činidla, v souladu se zásadami zelené chemie, přičemž funkční skupiny biopolymeru sloužily jako stabilizátory a katalyzátory tvorby polypyrrolu. Byly připraveny čtyři vzorky na bázi chitooligosacharidů (nonamer, oktamer, pentamer, dimer), které byly následně analyzovány.

Koloidy byly charakterizovány pomocí metody dynamického rozptylu světla (DLS), která ukázala, že delší řetězce chitooligosacharidů vedly k stabilnějším koloidům s nižším indexem polydisperzity a vyšším zeta potenciálem. UV/VIS spektroskopie potvrdila úspěšné zakomponování polypyrrolu do všech vzorků, s charakteristickými absorpčními maximy okolo 290 nm a 480 nm.

Cytokompatibilita byla vyhodnocena pomocí MTT testu na fibroblastech NIH/3T3 a makrofázích RAW 264.7. Vzorky s nonamerem a oktamerem vykazovaly vysokou buněčnou viabilitu, zatímco vzorek s pentamerem vykazoval koncentračně závislou cytotoxicitu. Dimer byl výrazně cytotoxický, zejména pro makrofágy. Imunomodulační účinky byly zkoumány prostřednictvím produkce oxidu dusnatého (NO) pomocí Griessova činidla a stanovení interleukinu-6 (IL-6) metodou ELISA u lipopolysacharidem (LPS) stimulovaných makrofágů. Vzorky OCH9_ppy a OCH8_ppy vedly k výraznému potlačení zánětlivé odpovědi, zatímco OCH5_ppy a OCH2_ppy vykazovaly mírnější účinky, pravděpodobně v důsledku jejich nižší stability a cytotoxického účinku.

Výsledky této práce ukazují, že délka řetězce chitooligosacharidů hraje zásadní roli při ovlivnění stability koloidů, biokompatibility a imunologické odpovědi. Vzorky s delšími řetězci byly vyhodnoceny jako nejvhodnější pro další aplikace v biomedicíně, např. hojení ran, cílené dodávání léčiv nebo tkáňové inženýrství.

Klíčová slova: koloidní částice, polypyrrol, chitooligosacharidy, dynamický rozptyl světla, biokompatibilita, imunomodulace

ABSTRACT

This thesis focuses on the synthesis, characterization, and biological evaluation of colloidal particles composed of polypyrrole and chitooligosaccharides of varying chain lengths. The particles were prepared using an oxidant-free method, in line with the principles of green chemistry, where the functional groups of the biopolymers acted as both stabilizers and catalysts for polypyrrole formation. Four different chitooligosaccharide-based samples (nonamer, octamer, pentamer, and dimer) were synthesized and analyzed.

The colloids were characterized using dynamic light scattering (DLS), which revealed that longer chain chitooligosaccharides resulted in more stable colloids, with lower polydispersity indexes and higher zeta potentials. UV/VIS spectroscopy confirmed successful incorporation of polypyrrole into all samples, with characteristic absorbance peaks at approximately 290 nm and 480 nm.

Cytocompatibility was assessed using the MTT assay on NIH/3T3 fibroblasts and RAW 264.7 macrophages. The nonamer and octamer exhibited high viability in both cell lines, while the pentamer showed dose-dependent cytotoxicity and the dimer was markedly cytotoxic, particularly to macrophages. Immunomodulatory effects were evaluated through nitrite production (Griess assay) and IL-6 secretion (ELISA) in lipopolysaccharide (LPS) stimulated macrophages. OCH9_ppy and OCH8_ppy significantly suppressed pro-inflammatory responses, while OCH5_ppy and OCH2_ppy showed moderate to weak suppression, likely influenced by their colloidal instability and cytotoxicity.

Overall, the results demonstrate that chitooligosaccharide chain length plays a crucial role in determining the colloidal behavior, biocompatibility, and immunological response of the synthesized particles. The longer-chain-based colloids were identified as the most promising for further application in biomedical fields such as wound healing, drug delivery, and tissue engineering.

Keywords: colloidal particles, polypyrrole, chitooligosaccharides, dynamic length scattering, biocompatibility, immunomodulation

I would like to thank Prof. Ing. Petr Humpolíček, Ph.D. for his guidance during the consultations on my Master's thesis. My gratitude also goes to Mgr. Jan Vícha, Ph.D. and Ing. Filip Latečka for their valuable advice and cooperation in the development and implementation of the analytical methodology.

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INTRODUCTION

Colloid systems represent a key tool in biomedicine. Their unique properties such as high surface-to-volume ratio, controllable particle size and the possibility of functional modification allow their broad use in targeted drug delivery, biosensors and tissue engineering. These systems, characterized by particles ranging from 1 nm to 1 μ m in size, offer distinctive optical, mechanical, and chemical properties that differ from both molecular solutions and bulk materials. Colloidal particles can exist in various forms, such as sols, emulsions, aerosols, and foams, and can be composed of solid, liquid, or gas phases dispersed in a continuous medium. This versatility makes colloidal systems highly suitable for a wide range of applications, including drug delivery, biosensing, environmental remediation and (bio)material design. (1–3)

Conductive polymers have gained significant attention due to their unique electrical properties, offering electrical conductivity to applications where interaction between electrical and biological systems is required. Among these, polypyrrole (PPy) stands out because of its ease of synthesis, environmental stability, and inherent conductivity. However, traditional methods of polypyrrole synthesis often involve the use of toxic oxidizing agents, which can limit the biocompatibility and sustainability of these materials. (4–6)

Biopolymers, such as chitosan, offer an attractive alternative to synthetic polymers due to their natural origin. Derived from the shells of crustaceans, chitosan is a linear polysaccharide, composed of D-glucosamine and N-acetylglucosamine units, that exhibits a range of desirable properties, including antimicrobial activity, biodegradability, and ease of modification. When combined with conductive polymers, biopolymers such as chitosan enhance the sustainability and biocompatibility of the resulting composite materials, which is crucial for biomedical and environmental applications. (7–10)

I. THEORETICAL PART

1 COLLOIDAL SYSTEMS

In a true solution, such as sugar or salt dissolved in water, the solute particles are dispersed in the solvent as individual molecules or ions, with particle sizes ranging from 0.1 nm to 1 nm. (1, 11)

For a colloidal system, the IUPAC definition states that it consists of two phases: a dispersed phase and a dispersant phase. The dispersed phase forms particles ranging in size from 1 nm to 1000 nm, with at least one dimension within this range. These microscopic particles can be created either by the bottom-up approach, *i. e.* by aggregation of small molecules into larger complexes, or by the top-down approach, meaning by the breakdown of bulk materials into smaller particles. (1, 2, 12, 13)

The dispersed phase can consist of substances like silica, aluminum or manganese oxides, or macromolecular biopolymers, such as polysaccharides, starches, proteins, or even whole blood cells. The continuous dispersant phase can be in a gaseous, liquid, or solid state. (1, 2, 13)

Due to their larger particles, when compared to a true solution, colloidal particles exhibit specific behaviors in a liquid medium, such as Brownian motion, diffusion, and a noticeable sedimentation rate. Colloids also display optical properties, including the Tyndall effect and light scattering by particles, which also helps to distinguish them from true solutions. Colloidal particles have a large ratio of the surface area to volume. Furthermore, due to their charge transfer properties, colloids exhibit electrical properties like electrokinetic potential and electroosmosis. The wide variation in particle size, molar masses, and electrokinetic potential among colloids results in polydispersity. Therefore, because of their complexity, colloidal systems require complementary analytical techniques for characterization and component analysis. (1, 2, 14)

Given that the dispersed phase and a dispersion medium may exist in gaseous, liquid, or solid states, several types of colloidal systems can be theoretically formed. Various types with examples are listed in Table 1. (1)

Table 1 Types of colloidal dispersions

Type name	Dispersed phase	Dispersion medium	Examples
Foam	Gas	Liquid	Whipped cream, shaving cream
Solid foam	Gas	Solid	Foam rubber
Aerosol	Liquid	Gas	Fog, mist
Emulsion	Liquid	Liquid	Milk
Solid emulsion (gel)	Liquid	Solid	Butter
Smoke	Solid	Gas	Dust, soot in air
Sol	Solid	Liquid	Paint, ink
Solid sol	Solid	Solid	Alloys

Source: (1)

Colloidal particles absorb light energy and scatter it in all directions. This phenomenon is called the Tyndall effect and the visible light beam or cone scattered by the dispersed phase is referred to as the Tyndall beam/cone. If the particles are large enough, the system may (as a result of the Tyndall effect) appear turbid under normal lighting conditions. Since true solutions do not show this effect, it can be used to distinguish true solutions from colloids. (1, 15)

1.1 Stability of colloidal systems

The application of a given colloidal system is dependent on its stability. Since colloidal systems are typically not true solutions, but rather homogeneous mixtures of two or more substances, they are not stable over time. After a period, the dispersed phase may separate from the continuous phase and form aggregates or flocks. This phase separation alters the properties of the system. Therefore, maintaining the stability of colloidal systems is crucial. Stability can be classified into various types, including kinetic, thermodynamic, electrostatic, and steric (depletion) stability. (13, 14, 16)

Brownian movement helps counteract the settling of colloidal particles caused by gravity. A colloidal particle is being constantly struck by the molecules of the dispersion medium from opposite directions, resulting in a rapid zig-zag motion. This movement has

a stirring effect and thus contributes to the kinetic stability of the system. It furthermore distinguishes colloidal systems from true solutions and suspensions. (17, 18)

Kinetic stability is linked to the presence of an energy barrier that prevents particles from coagulating. Thermodynamic stability, on the other hand, refers to a scenario where the coagulated phase has a higher free energy than the dispersed phase. Electrostatic stabilization arises from electrostatic repulsion forces. When particles suspended in a liquid medium carry the same electrostatic charge on their surfaces, they repel each other, which further enhances stability. However, this type of stability is only effective in systems where the range of electrostatic repulsion is greater than the range of attractive forces, and it is more applicable to systems containing small particles. Additionally, it is generally considered to be short-term. The final type of stability is steric stability, which is achieved by adding macromolecules to the system. These macromolecules, such as surfactants or polymers, affect the stability process by altering the interphase properties of the dispersed particles. When they are added, the stability of the colloidal suspension depends on the specific stabilization mechanism involved. (16, 19, 20)

The stability of colloidal systems can be further explained by the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory. This theory defines stability in terms of the interaction energy between particles in the solution. The interactions between the particles can be divided into two main forces: van der Waals attraction forces and repulsive electric double layer forces. If the repulsive forces exceed the attractive forces, the system is considered stable (Figure 1). There are three types of attractive interactions: the permanent dipole-permanent dipole (Keesom), permanent dipole-induced dipole (Debye) and transient dipole-transient dipole (London). If they are weaker than the repulsive forces, particles tend to adhere to each other, leading to flocculation, which is the opposite of stabilization. The addition of various substances, as mentioned above, as well as factors such as concentration, ionic strength, or pH, can alter the total potential energy of these interactions. (16, 19–23)

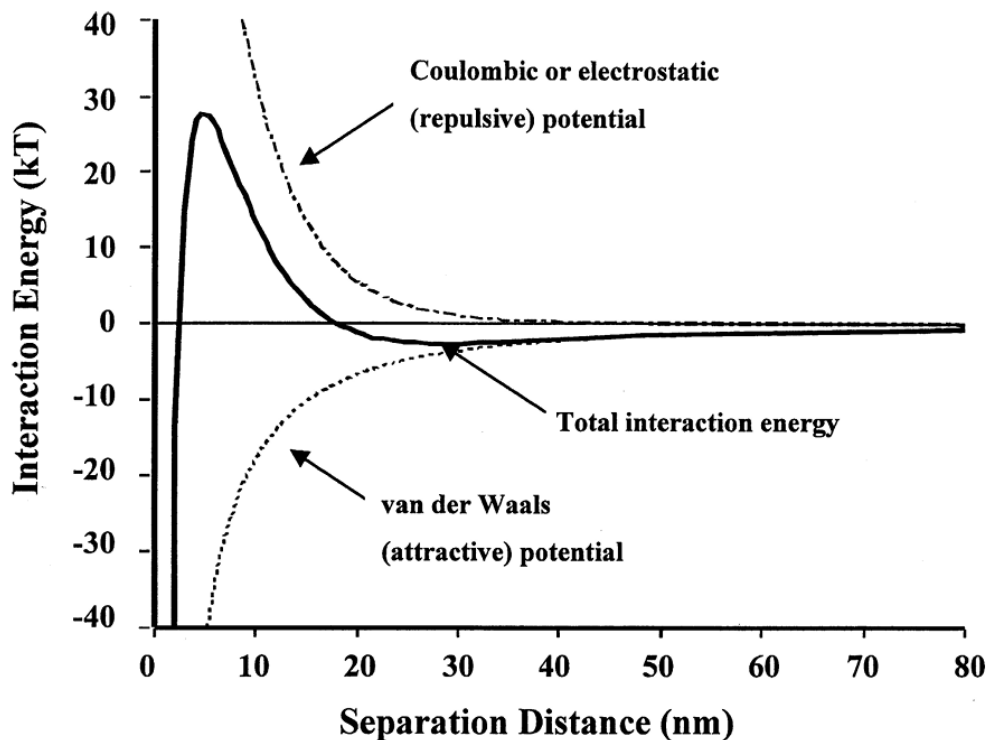


Figure 1 Schematic of interaction energy versus distance profiles of DLVO interaction. The attractive van der Waals and the repulsive electrostatic potentials form the total interaction energy.

Source: (20)

1.1.1 Stabilization mechanisms

Different stabilization mechanisms can be categorized as steric stabilization, electrosteric stabilization, and depletion stabilization. (20, 24)

Steric and electrosteric stabilization apply when a polymer is attached to the surface of a particle. In steric stabilization, the adsorbed polymer does not carry an electrostatic charge. Electrosteric stabilization, on the other hand, refers to a system where the polymer is ionic in nature. Long-chain molecules are anchored to the surface of the particles, creating a thin shell or envelope of these molecules around each particle (Figure 2.). The anchoring of these long-chain molecules can be achieved through chemical synthesis (also known as chemical grafting) or by adsorption. (20, 24)

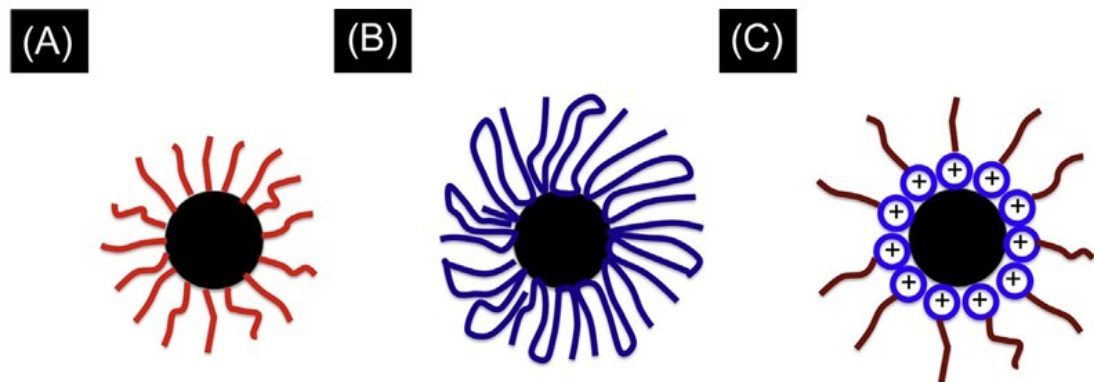


Figure 2 Schematic representation of steric and electrosteric stabilization of a particle due to (A) covalently attached long-chain molecules, (B) adsorption of long chain molecules such as polymers, and (C) adsorption of surfactant molecules where the tail is exposed to the dispersant phase.

Source: (24)

The depletion stabilization mechanism occurs when the polymer is not adsorbed onto the dispersed particles. Instead, free polymer chains, forming various structures, are positioned between the solid particles. This reduces the attraction between the particles, influencing the system stability. (16, 20)

Depletion stabilization therefore involves adding nonionic polymers to a colloidal dispersion. When particles in a polymer solution come close to one another, the polymer molecules between the particles are generally expected to leave the gap between the particles. In other words, the polymer is "depleted" from the space between the particles. However, if the interaction between the polymer and the solvent is more favorable (*i.e.*, the polymer is in a good solvent), the polymer remains in the region between the particles because that is energetically more advantageous. Therefore, the polymer molecules continue to occupy the space between the particles, providing stability to the colloidal dispersion through depletion stabilization. (16, 20, 24)

1.1.2 The electrical double layer

Colloidal particles can be engineered by attaching one or more chemical groups to the molecules or atoms on the particle surface. When these particles are dispersed in a liquid, the chemical groups on the particle surface may dissociate, resulting in a charged group on the particle surface. This layer of ions selectively attracts counter-ions from the surrounding dispersion medium, forming a second layer of charges, that balances the charge on the dispersed particles. The layer of counter-ions adjoining closely the charged surface is called

the Stern layer. These ions are bound to the charged surface and are expected to move with the particle when it is in motion. To ensure that the system is electroneutral as a whole, the total charge on all the colloidal particles in the dispersion must be equal but opposite to the total charge of all the counter-ions. The combination of these two layers, meaning the positive and negative charges around the dispersed particle, is known as the Helmholtz Double Layer. The term Stern Double Layer refers to a more recent concept of a compact layer of ions and counterions fixed firmly on the particle surface, and the diffuse layer of counterions that is only loosely attached to the particle's surface and moves in the opposite direction when electric field is applied (Figure 3). (1, 17, 19, 24–26)

The difference in potential between the compact and diffuse layers is referred to as Zeta potential or Electrokinetic potential. It is caused by the distribution of the charge around the particle and is dependant on several factors, such as solvent pH, conductivity, temperature, and presence, type and concentration of ions. Zeta potential measurements can provide useful information about the electrostatic stability of a colloid system. (17, 26–28)

When a charged particle is subjected to a disturbance, such as an externally applied electric field, or when an electrolyte is added to a dispersion of charged colloidal particles, the free ions in the solution redistribute, leading to the restructuring of the electrical double layer. (24, 26, 28)

Repulsive interactions between colloidal particles occur when the electrical double layers of neighboring particles overlap. (24, 26)

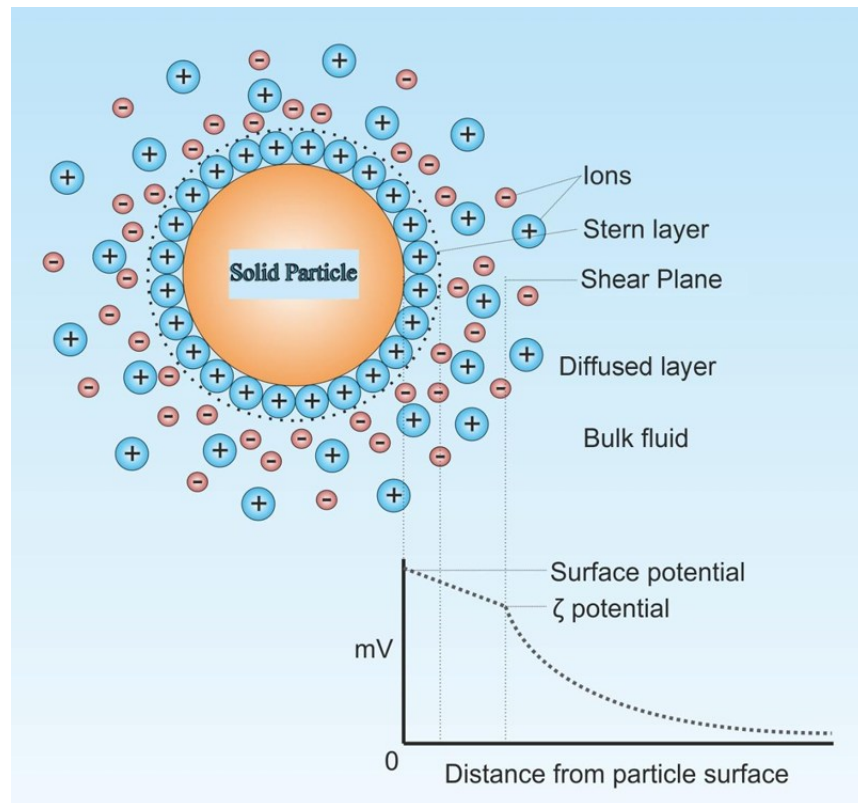


Figure 3 Schematic representation of an electrical double layer on a charged spherical particle

Source: (28)

1.2 Characterization methods of colloidal systems

As colloidal systems become more widely used in manufacturing and technology, a growing need for improving the ways we study and analyse them arises. Whether to better understand the properties of individual particles, such as their size and distribution, or to examine how they interact with other components of the system, such as polymers and surfactants. (2, 18)

1.2.1 Microscopy techniques

Numerous techniques are available for determining particle dimensions and size distribution. In many cases, a combination of different methods is required to obtain accurate and comprehensive results. While advanced techniques, such as those based on specific types of microscopy or light scattering, can provide detailed and precise measurements, simpler methods, including sedimentation or backscattering, may be more suitable for continuous particle size monitoring. Figure 4 shows the resolution and some characteristics and applications of these advanced methods. (29, 30)

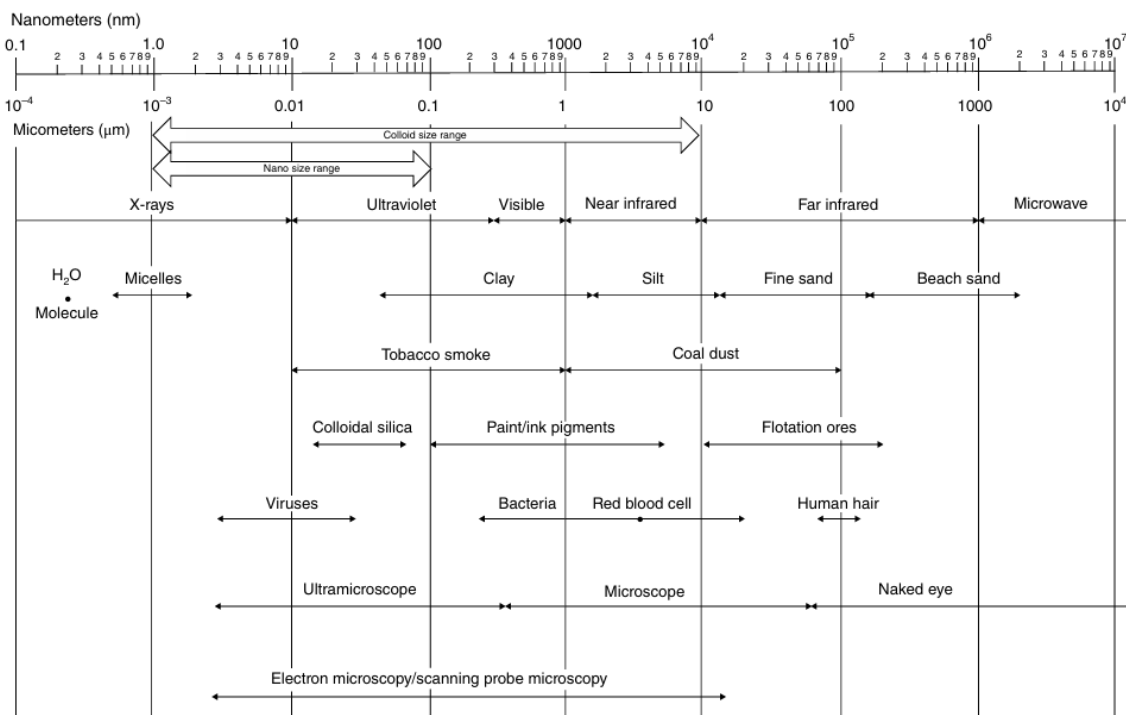


Figure 4 Size estimations of common particles as well as methods for their characterization.

Source: (28)

Optical microscopy includes direct visual techniques used to examine the structure and surface characteristics of particles or droplets. This method relies on detecting light that is reflected, refracted, or emitted as fluorescence from the investigated sample. Traditional optical microscopy is limited to observing the upper size range of colloidal particles, offering a typical resolution between 0.5 and 1 μm . The maximum magnification achievable with conventional optical microscopes is approximately 1000 $\times$. Additionally, the depth of field, defined as the range within which the image remains in acceptable focus, is typically around 1 mm. (29, 30)

The two most widely used techniques for high-resolution imaging in this field are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM, is a powerful method for examining surface structures at extremely high magnifications, typically ranging from 10 $\times$ to 500 000 $\times$. Traditionally, it requires extensive sample preparation, such as carbon or gold sputtering, and vacuum conditions. However, advancements in instrumentation have led to the development of environmental SEM (E-SEM), which utilizes a pressure-controlled chamber and reduced working distances. This allows for direct observation of samples without the need for prior coating or vacuum conditions. (29–31)

TEM operates by directing a beam of electrons through an ultrathin specimen, during which the electrons interact with the material as they pass through it. From these interactions, an image is formed, then magnified and recorded. TEM offers exceptionally high magnification and resolution. It is capable of visualizing even a single row of atoms. Due to this level of detail, TEM is widely used in various research areas, including cancer biology, catalysis, and materials science, among many other scientific and industrial applications. (13, 29, 30, 32)

Another one of the most widely utilized techniques for high-resolution surface analysis is atomic force microscopy (AFM). AFM operates using an extremely sharp tip that scans the surface of the sample. The topographical image is generated by detecting the forces between the tip and the sample surface as the tip moves across it. A key advantage of AFM over techniques such as TEM is that measurements do not need to be conducted in a vacuum. Imaging can occur in air or even in liquid environments. Furthermore, sample preparation is minimal, as there is no need for conductive coatings or staining. However, the resulting image is a merged effect of both surface topography and tip dynamics, therefore its interpretation requires consideration of these factors. (29, 30)

1.2.2 Light scattering techniques

As stated above, colloids have the capacity to scatter light, often exhibiting pronounced turbidity. The intensity, angular distribution, and polarization of the scattered light depend on several factors, including the size and shape of the particles, their interactions, and the refractive index contrast between the particles and the surrounding medium. These principles are used in light scattering techniques, which are widely employed to determine particle size distributions in colloidal systems. These methods are rapid, suitable for both dry and wet samples, and require minimal sample preparation. Since scattering intensity increases with particle size, such techniques are also valuable for monitoring particle aggregation (coagulation) and evaluating the stability of colloidal dispersions. (27, 31)

Techniques such as small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS) are commonly used to analyze particle size distributions and aggregation behavior. Computer simulations, including Monte Carlo methods and molecular dynamics, also provide valuable insights into aggregation mechanisms. However, each method has limitations. SAXS, for instance, requires complex data analysis and mathematical modeling, while Monte Carlo simulations are resource-demanding and typically require validation

through experimental data. In contrast, DLS is widely favored in both industrial and research settings due to its simplicity, speed, and potential to measure other colloidal properties, such as Zeta potential by measuring a charged particle's electrophoretic mobility. (13, 27)

Particles in suspension undergo random motion, known as Brownian motion, which is influenced by their size. Smaller particles move faster, while larger particles move more slowly. Dynamic Light Scattering (DLS) uses a laser to illuminate particles and analyzes intensity variations in the scattered light to relate this motion to particles' hydrodynamic size. Large particles, moving slowly, show gradual fluctuations in intensity, while small particles, moving quickly, cause rapid intensity changes. Therefore, as the laser illuminates dispersed nanoparticles, they scatter light according to their size, while the particles' continuous motion causes fluctuations in the scattered light's phase, which are observed as intensity variations. DLS measures the rate of these intensity fluctuations and uses the Stokes–Einstein equation (Equation 1) to connect particle size with the speed of Brownian motion. (2, 27, 31)

$$D = \frac{kT}{f} = \frac{kT}{6\pi\eta R_H} \quad (1)$$

where D is translational diffusion coefficient, k is Boltzmann constant, T is absolute temperature, R_H hydrodynamic radius and η dynamic viscosity. (2)

2 CONDUCTING POLYMERS

Electroactive biomaterials represent a type of smart materials capable of delivering electrical, electrochemical, and electromechanical stimulation directly to cells. Bioelectricity is a fundamental process in mammalian and human cells. Consequently, intrinsically conductive polymers are extensively studied for their potential use in biosensors and as cell-instructive materials in regenerative medicine. This category of biomaterials includes conductive polymers, electrets, piezoelectric, and photovoltaic materials. While electrets and piezoelectric materials can deliver electrical stimuli without requiring an external power source, their control over the stimulus is limited. In contrast, conductive polymers offer excellent control over electrical stimuli, have superior electrical and optical properties, exhibit a high conductivity-to-weight ratio, and can be designed to be biocompatible and biodegradable. (4, 5, 12)

Early research on conducting polymers was initiated by the discovery of conductivity of polyacetylene, a polymer that is typically only semiconducting. Its conductivity increases significantly when oxidized with iodine vapor. This ability to manipulate the polymer's conductivity, known as doping, opens up a range of applications in electronics, biomaterials, and energy storage systems. (4, 6)

A significant advantage of conductive polymers is their versatility; their chemical, electrical, and physical properties can be customized to meet the specific requirements of an application by incorporating antibodies, enzymes, and other biological components. These materials combine the advantageous properties of metals, such as charge conduction, with the processing flexibility and easy synthesis of traditional polymers. Moreover, these properties can be adjusted and controlled through external stimuli (*e.g.*, electricity, light, pH) even after the polymers have been synthesized. Common examples of conducting polymers include polypyrrole (PPy), polyacetylene (PA), polyphenylene, polyaniline (PANI), poly(3,4-ethylenedioxythiophene) (PEDOT) (Figure 5), polythiophene (PTH), poly(phenylenevinylene) (PPV), and polyfuran (PF). (4, 33–35)

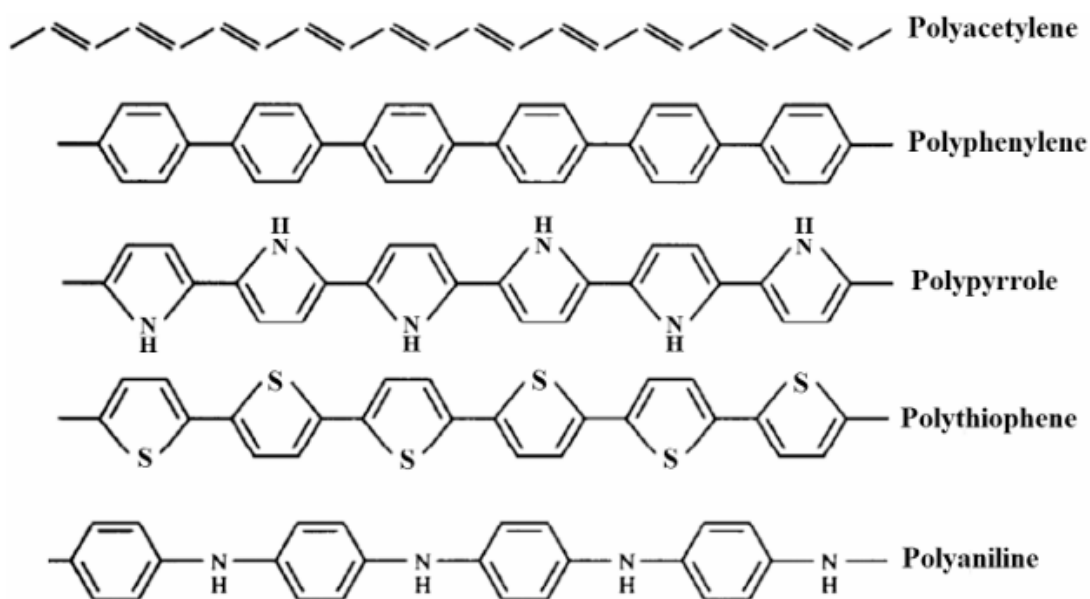


Figure 5 Chemical structures of polyacetylene (PA), polyphenylene, polypyrrole (PPy), polythiophene (PTH) and polyaniline (PANI)

Source: (35)

A conjugated carbon chain is characterized by alternating single and double bonds. While both the single and double bonds have a strong, localized σ -bond, the double bonds also include a less localized π -bond. The π -orbitals in these bonds overlap, allowing electrons to be more delocalized, meaning they can move freely across multiple atoms rather than being fixed to a single one. This delocalization is critical for the formation of charge carriers such as polarons, bipolarons, and solitons, which are key to the transition of the material from an insulator to a conductor. (4, 6, 36)

Unlike conventional polymers, which consist of thousands to millions of monomer units and are typically stiff and soluble in solvents, a conjugated polymer chain is made up of fewer monomer units. The solubility and processability of conducting polymers are primarily influenced by the side chains attached to the polymer and the dopant ions, which grant the polymers with mechanical, electrical, and optical characteristics. Conducting polymers exhibit both crystalline and partially amorphous structures. (6, 37)

2.1 Synthesis methods

Currently, there are two primary methods for synthesising conductive polymers: chemical and electrochemical. (4, 35, 37)

2.1.1 Chemical synthesis

In chemical synthesis, a monomer solution is combined with an oxidizing agent (e.g., ferric chloride, ammonium persulfate). This process leads to the formation of either a powder or thick film of the polymer, enabling bulk production and thus making it a preferred method for commercial applications. An advantage of chemical polymerization is its versatility, allowing the synthesis of various types of conductive polymers, including some that cannot be created through electrochemical methods. However, the conductivity of polymers produced via chemical synthesis tends to be lower than those produced electrochemically. Additionally, the conductivity of the resulting polymer is highly sensitive to factors like the solvent type, oxidant purity, reagent concentration, reaction time, temperature, and stirring rate, making it difficult to achieve reliable and consistent results. (4, 5, 37)

2.1.2 Electrochemical synthesis

Electrochemical polymerization involves applying an electrical current through electrodes submerged in a solution containing the monomer, solvent, and doping agent (Figure 6). This technique enables the deposition of thin films of polymer with controlled thickness (down to 20 nm) and morphology. The electrical current causes the monomer to oxidize and deposit on the positively charged electrode, forming insoluble polymer chains. The properties of the resulting film are influenced by factors such as deposition charge, time, temperature, solvent, doping agent, and electrode system. Electrochemical polymerization can only be used for polymers whose monomers can undergo oxidation in the presence of an electrical potential, such as PPy, PANI and PEDOT. (4, 20, 37, 38)

There are three main electrochemical polymerization techniques: galvanostatic, potentiostatic, and potentiodynamic. In potentiostatic polymerization, the potential of the electrodes is controlled while the current varies, making it ideal for applications like biosensors. The current is influenced by factors such as electrode material and plating conditions and coulometer is needed to control the deposition. In galvanostatic polymerization, the current is controlled instead of the potential, allowing precise control over the polymer deposition rate. During potentiodynamic polymerization, the polymerizing

potential is cycled between low and high limits, causing the polymer to deposit in layers, each becoming electrically active before the next layer is synthesized. Potentiodynamic electrosynthesis results in a different surface morphology compared to potentiostatic and galvanostatic methods. For example, when comparing PEDOT synthesized by all three techniques, the potentiostatic and galvanostatic methods produced a porous, globular surface, while the potentiodynamic method resulted in a rod-like, fibrous morphology. (4, 37, 38)

Electrochemical synthesis allows rapid in situ deposition of conductive polymers, but the amount of bioactive molecules that can be doped into the polymer is limited. Additionally, because the polymer must be deposited onto an electrode, the shape and size of the electrode restrict the range of geometries and quantities that can be synthesized. This limitation also makes it difficult to create composites using electrochemical synthesis. In contrast, chemical synthesis facilitates the easy creation of composites, and has been used to combine PPy with polymers such as poly(methyl methacrylate), poly(vinyl chloride), polystyrene, and polyurethane. (4, 37, 38)

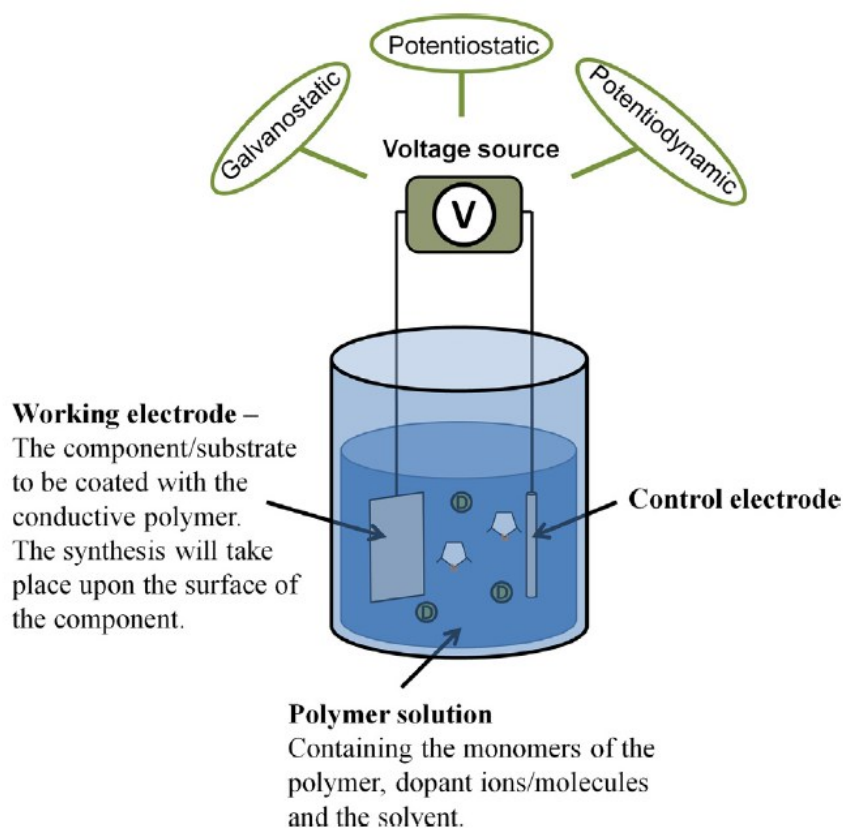


Figure 6 A schematic of the electrochemical synthesis set-up.

Source: (4)

2.2 The doping process

During the doping process, the neutral insulating polymer is transformed into an ionic complex, consisting of a polymeric cation (or anion) and a counterion, which is the reduced form of the oxidizing agent (or the oxidized form of the reducing agent). Therefore, a key factor in selecting potential conducting polymers is how easily the polymer can be oxidized or reduced. This also explains the preference for π -bonded unsaturated polymers, like polyacetylene, which have low ionization potentials, or large electron affinities. Electrons can be then relatively easily added or removed to form a polymeric ion, without significantly disrupting the bonds that are responsible for holding the polymer structure together. (39, 40)

Another key factor in conductivity is the dopant molecule. The polymer is initially synthesized in its oxidized, conductive state, and the dopant molecule (usually an anion) stabilizes the backbone and neutralizes the charge. Additionally, the dopant introduces charge carriers by removing or adding electrons from or to the polymer chain, forming polarons or bipolarons. When an electrical potential is applied, the dopants move in or out of the polymer, disturbing the stable backbone and enabling charge transfer in the form of polarons and bipolarons. (4, 41, 42)

The doping process is reversible, meaning that applying an electrical potential can cause the dopant to leave or re-enter the polymer, thus switching it between its conductive and insulating redox states. This is an important feature for various applications, including sensors, and other smart materials where the material's properties can be dynamically controlled. (6, 42)

2.2.1 Doping methods and dopant molecules

There are two primary types of doping. During the process of p-doping, the polymer is oxidized and carries a positive charge, on the other hand, n-doping produces a reduced polymer that carries a negative charge. (6, 42)

Doping typically happens during the synthesis of the polymer and can be achieved through different methods: chemical doping, electrochemical doping, or photodoping. Electrochemical doping is often the preferred method, particularly for when the dopant is a biological molecule. In this case, the biological molecule is charged and incorporated into the polymer during the electrochemical polymerization process. This is important because

many biological molecules cannot undergo the necessary redox chemistry for chemical doping. (6)

The amount of dopant used is directly proportional to the resulting conductivity of the doped polymer. The more dopant added, the higher the conductivity. Typically, the maximum dopant concentrations are around several mole percent per repeat unit of the chain backbone. For example, polyacetylene doped with iodine can reach up to 20% of a dopant concentration. However, the choice of a dopant molecule not only influences the conductivity but also affects other structural properties of the polymer, such as its color, porosity, and volume. (6, 39)

Dopants can be categorized into two groups based on their molecular size. Small dopants (*e.g.*, Cl^-) and large dopants (*e.g.*, sodium poly(styrene sulfonate), PSS). Both types affect the polymer's conductivity and structural properties in different ways. (35, 38, 39)

Small dopants are inherently more mobile within the polymer and can easily leave and re-enter the material when subjected to electrical stimulation. This reversibility makes them particularly useful in applications such as drug release systems, where the dopant can be removed and replaced to control the release of a therapeutic molecule. Additionally, small dopants allow the physical properties of the polymer to be controlled through repeated cycling between doping (oxidation) and dedoping (reduction). (35, 39)

Large dopants are more integrated into the polymer's structure and have a more pronounced effect on its properties. They tend to increase the density of the polymer and also improve the electrochemical stability since they are less likely to be leached out over time, or by the application of an electrical stimulus. (38, 39)

The choice of a dopant molecule significantly influences the bulk and surface properties of the polymer. Surface properties such as roughness, wettability, and stiffness are known to affect the adhesion and proliferation of various cell types. (4, 35, 38)

Different dopants can modify these properties in diverse ways. As mentioned above, biological molecules, such as enzymes, proteins and antibodies have been used as dopants in conductive polymers. For example, according to Balint *et al.*, hyaluronic acid doped polypyrrol makes the material more brittle and rough, doping polypyrrol with chondroitin sulfate results in a material with an increased surface roughness, whereas PEDOT has been doped with hyaluronic acid, heparin or fibrinogen for the purpose of increasing its biocompatibility. (4, 42)

While doping with biomolecules can improve the properties of conductive polymers, it can also introduce some challenges such as compromising the polymer's integrity or possibly reducing its conductivity, highlighting the potential trade-off between biocompatibility and conductivity. Thus, careful selection of dopants is essential for balancing the desired material properties, such as mechanical strength or biocompatibility, for specific applications. (4, 35, 42)

2.3 Polypyrrole

Polypyrrole (PPy) is probably the most extensively studied conductive polymer. It exhibits a wide range of excellent characteristics and stimulus-responsive properties, making it a promising smart biomaterial. It demonstrates good biocompatibility both *in vitro* and *in vivo*, sufficient chemical stability in environments such as air and water and relatively high conductivity under physiological conditions. Furthermore, it is easily and flexibly synthesized at room temperature in large quantities, using a variety of solvents, including water. Its properties can also be modified for specific biomedical applications through the incorporation of bioactive molecules. Additionally, PPy is stimulus-responsive, allowing dynamic control of its properties through the application of an electrical potential. (4, 12, 42)

Oxidized polypyrrole carries a positively charged backbone, which necessitates the incorporation of anionic counterions to maintain charge neutrality. Charge transport occurs primarily through bipolarons, which are more mobile than polarons and thus serve as the dominant charge carriers. The electrical and structural properties of PPy are closely linked to the synthesis method, such as electropolymerization or chemical polymerization. Polypyrrole, however, also has its limitations. Once synthesized, it becomes difficult to further process due to its molecular structure, which makes it mechanically rigid, brittle, and insoluble. Despite these challenges, PPy is used in a wide range of applications, including fuel cells, corrosion protection, microsurgical tools, biosensors, and drug delivery systems. Furthermore, PPy is the most frequently reported conductive polymer incorporated into skin tissue engineering scaffolds or membranes. It also serves as a biomaterial in neural tissue engineering, and is used for neural probes, nerve guidance channels, blood conduits and biosensors. (4, 12, 36, 37, 42)

2.4 Polyaniline

The second most studied conductive polymer after polypyrrole is polyaniline, also known as aniline black. It exists in several forms depending on its oxidation state: the fully oxidized pernigraniline base, the half-oxidized emeraldine base, and the fully reduced leucoemeraldine base. Among these, the emeraldine base is the most stable and conductive. (4, 41)

PANI offers several advantages, including ease of synthesis, low cost, good environmental stability, and the ability to electrically switch between its conductive and resistive states. However, its application in biological contexts is limited due to its poor processability, lack of flexibility, non-biodegradability, and tendency to cause chronic inflammation once implanted. Despite these challenges, PANI has been explored for use in biosensors, neural probes, controlled drug delivery systems, and tissue engineering applications. (4, 41)

2.5 Polyacetylene

The invention of polyacetylene and its enhancement in conductivity through doping was recognized with the Nobel Prize. Polyacetylene and its derivatives exhibit multifunctional behaviors, including electrical conductivity and photoconductivity. The main chain of polyacetylene is made up of a flexible linear polyene chain, which can be decorated with pendant groups by replacing the hydrogen molecules in the alternating carbon structure with foreign molecules, creating monosubstituted or disubstituted polyacetylene. (6, 42, 43)

There are several methods for synthesizing polyacetylene: catalytic polymerization, non-catalytic polymerization, catalytic polymerization of other polymers, and precursor-assisted synthesis. In catalytic polymerization, catalysts such as Ziegler–Natta catalysts or Luttinger catalysts are used. Ziegler–Natta catalysts are highly soluble in organic solvents and provide high selectivity, producing highly crystalline, free-standing polyacetylene films. Luttinger catalysts, consisting of a hybrid reducing agent and a metal complex, are also used for polyacetylene synthesis. They typically produce polyacetylene with a high molecular weight and fewer oligomers, although they are less catalytically active than Ziegler–Natta catalysts. (6, 44)

Electrochemical polymerization is a non-catalytic polymerization method. This involves anodic oxidation of a monomer precursor in the presence of suitable electrolytes on

an inert metal surface, forming polyacetylene films. Additionally, light-induced synthesis using UV irradiation of acetylene gas has been explored for producing polyacetylene. (6, 44)

These diverse synthesis methods offer flexibility in tailoring the properties of polyacetylene for specific applications, including its use in electronics, sensors, and other advanced materials. (6, 43, 44)

2.6 Applications of conducting polymers in biomedicine

Conducting polymers have emerged as a unique class of functional materials with growing relevance in biomedical applications. Their ability to conduct electrical signals, combined with tunable mechanical properties, surface functionality, and chemical versatility, makes them particularly well-suited for interfacing with biological systems. Among the most studied are polypyrrole, polyaniline, and poly(3,4-ethylenedioxythiophene) (PEDOT), which exhibit excellent electrochemical properties and can be synthesized under mild, biocompatible conditions. (4, 12, 36)

In biomedicine, these materials are being investigated for use in biosensors, drug delivery systems, neural interfaces, tissue engineering scaffolds, and wound healing materials. Their electrical conductivity allows for cell stimulation, signal transduction, and responsive behavior, enabling dynamic interaction with living tissues. Additionally, conducting polymers can be engineered to support cell adhesion, proliferation, and differentiation, further enhancing their potential as biofunctional materials. (12, 37, 42)

2.6.1 Wound healing and antimicrobial activity

Conductive polymers are being explored as an innovative strategy for accelerating wound healing, enhancing antibacterial activity, and enabling controlled drug release as they facilitate direct electrical stimulation at the wound site. A skin wound is characterized as the disruption of the skin's normal anatomical structure and function. The healing process consists of several distinct phases: hemostasis, inflammation, proliferation, maturation, and remodeling. (4, 36)

Electrical stimulation in wound care involves applying an external electrical stimulus to the tissue adjacent to or within a wound. This technique aims to enhance control over cellular differentiation and proliferation, thereby accelerating the wound healing process. The underlying mechanism is based on the natural transepithelial potential maintained by healthy skin. When skin integrity is compromised, this potential is disrupted, creating an

electric field at the wound edges that guides cell migration toward the wound center and influences both the orientation and rate of cell division. Incorporating conducting polymers into wound dressings and tissue scaffolds enables more consistent and targeted delivery of electrical stimulation to injured tissues. Interestingly, some studies have also reported that their sole use, without external stimulation, can independently promote faster wound healing. (4, 36, 45)

To enhance biocompatibility and physical performance, conducting polymers are often combined with biomolecules or biocompatible polymers through doping, composite formation, copolymerization, or blending. Overall, they show strong potential for advancing wound healing strategies and applications in skin tissue engineering. The doping capability of conductive polymers allows for the incorporation of specific therapeutic agents, enabling the controlled release of drugs directly at the wound site. This controlled drug delivery can be precisely regulated through the application of an external electrical field, offering targeted and responsive treatment. (36, 42, 45)

Moreover, conductive polymers and their composites have demonstrated inherent antibacterial properties. For instance, it has been reported that polyaniline exhibits antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa*, including both wild-type and antibiotic-resistant strains. Similarly, a polypyrrole/dextrin composite showed effective antibacterial action against *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*, bacteria frequently implicated in infected wounds. (4, 12)

2.6.2 Tissue engineering

Conductive polymers have also demonstrated significant potential in various tissue engineering applications due to their electrical conductivity, biocompatibility, and ease of synthesis. Materials, such as polyaniline, polypyrrole, and polythiophene, have been employed in scaffolds for bone, skeletal muscle, nerve, and cardiac tissue engineering. In bone tissue engineering, they have been used to promote osteogenesis and support bone cell growth through electrical stimulation. For skeletal muscle regeneration, conductive polymer-based scaffolds enhance myoblast alignment and differentiation. In nerve tissue engineering, they provide conductive pathways that support neuronal growth and nerve regeneration. In cardiac tissue engineering, conductive polymers facilitate the development of scaffolds that support cardiomyocyte function and electrical coupling necessary for heart tissue regeneration. Additionally, conductive hydrogels and composites have been shown to

accelerate wound healing by stimulating cellular activities through electrical signals. Overall, the ability of conductive polymers to interact with cells via electrical cues makes them versatile materials for enhancing tissue regeneration in a wide range of biomedical applications. (36, 42, 45, 46)

3 BIOPOLYMERS

Biopolymers are polymers produced by living organisms, including plants, animals, and microorganisms. The term "biopolymer" reflects both their biological origin and their biodegradable nature. (7, 8)

3.1 Structure, properties and applications of biopolymers

Structurally, biopolymers are composed of repeated units (monomers) covalently bonded together, often forming complex and versatile materials. Examples of natural biopolymers include rubber, starch, cellulose, proteins, DNA, RNA, chitin, and peptides. These materials play essential roles in nature by enabling energy storage, preserving and transmitting genetic information, and cellular construction. (7, 8, 47, 48)

Biopolymers offer significant advantages due to their biodegradability and biocompatibility. For instance, sugar-based biopolymers like polylactides can naturally degrade in the human body without producing harmful side effects, making them highly valuable for medical applications such as implants and drug delivery systems. Starch-based biopolymers can be processed through extrusion and injection molding to create environmentally friendly alternatives to conventional plastics. Similarly, cellulose-based biopolymers, such as cellophane, are used to manufacture packaging materials like thin wrapping films, food trays, and protective shipping pellets. (7, 8, 49–52)

In industry, biopolymers find broad applications across pharmaceuticals, food production, cosmetics, packaging, and even the petroleum sector. Synthetic biopolymers are also utilized in the fabrication of textiles and mats. (7, 8, 47, 50, 53)

Due to their sustainability and wide range of functional properties, biopolymers are considered key materials for the future, supporting the shift toward more eco-friendly technologies and products. (7, 47, 54)

3.2 Classification of biopolymers

Biopolymers can be categorized into four main groups according to their origin, the first three being derived from renewable resources: polymers from biomass (*e. g.* starch and cellulose), polymers produced by microbial synthesis (poly(hydroxy alkanoates)), polymers chemically synthesized from biological monomers (polylactid acid) and polymers of

synthetic origin, that are not naturally occurring, but designed to mimic natural structures and properties, often using biological raw materials as a starting point. (7, 48, 54, 55)

Based on the chemical structures synthesized by living organisms, polymers can be classified into three major categories: polysaccharides, proteins, and polynucleotides. (8, 48)

3.3 Biocompatibility and biodegradability

Biodegradability is regarded as one of the most critical characteristics of biopolymers. After disposal, these materials naturally degrade in environments such as soil, activated sludge, freshwater, and seawater. Numerous prokaryotic and eukaryotic microorganisms secrete extracellular depolymerases capable of hydrolyzing biopolymers, breaking them down into smaller compounds that can be used as nutrients. Eventually, these degraded compounds are converted into renewable resources, such as carbon dioxide (CO₂) and biomass. (7, 53)

The initial step in biopolymer recycling involves the enzymatic decomposition of polymers into oligomers and/or monomers by depolymerases. Given the importance of biopolymer stability and controlled degradation after disposal, extensive research has focused on understanding the biodegradation mechanisms of various biopolymers, including polyesters, polysaccharides, proteins, lignins, and related materials. (7, 48, 53, 56)

Some biopolymers can be produced from biomass or other renewable resources, however, not all of them are biodegradable. Non-biodegradable bio-based biopolymers include: synthetic polymers derived from renewable resources, such as specific polyamides from castor oil (*e. g.* polyamide 11), bio-based polyethylene (bio-LDPE, bio-HDPE), bio-based polypropylene (bio-PP), and bio-based poly(vinyl chloride) (bio-PVC) and naturally occurring biopolymers, such as natural rubber and amber. (8, 57)

3.4 Chitosan

Chitosan is a naturally occurring, amino-functional polysaccharide derived from the partial deacetylation of chitin. Structurally, it is composed of D-glucosamine and N-acetyl-D-glucosamine units linked via β -(1 $\rightarrow$ 4) glycosidic bonds. The presence of hydroxyl and amino groups in its backbone enables various chemical modifications such as esterification, etherification, and reductive amination, which are key to tailoring its physicochemical properties. The chemical structure of chitosan is depicted in Figure 8. (7, 9, 58, 59)

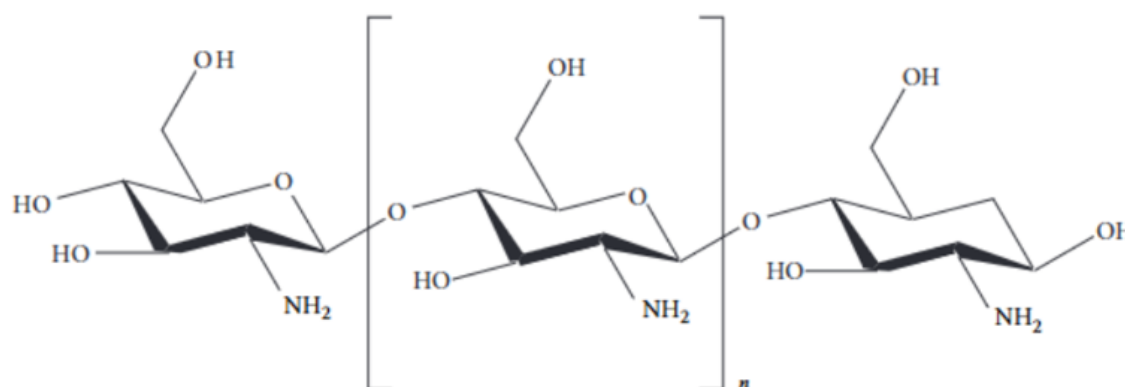


Figure 7 The chemical structure of chitosan.

Source: (58)

Chitosan is valued for its non-toxic, biocompatible, and biodegradable nature, which has led to extensive research in biomedical, pharmaceutical, and industrial applications. Its intrinsic properties, mucoadhesion, antimicrobial activity, and permeability enhancement, are closely related to the functionality of its amino and hydroxyl groups. Under acidic conditions, chitosan becomes cationic due to the protonation of amino groups, allowing it to interact electrostatically with negatively charged mucosal surfaces and cell membranes, facilitating mucoadhesion and paracellular transport through the opening of tight junctions. These interactions are further reinforced by hydrogen bonding and hydrophobic effects. (9, 58)

The degree of deacetylation and molecular weight are crucial in determining its solubility, viscosity, and biological activity. Native chitosan's poor solubility in neutral and basic media limits its broader use. To overcome this, various derivatives have been developed to improve solubility and functionality, relying on chemical modifications targeting its amino ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) groups. These modifications improve solubility, mucoadhesion, pH sensitivity, and drug permeation, thereby broadening its utility in drug delivery. Derivatives such as trimethyl chitosan, thiolated chitosan, glycol chitosan, and carboxymethyl chitosan exhibit enhanced biocompatibility, biodegradability, and effectiveness as drug carriers, particularly in controlled and targeted delivery systems. (9, 56)

Chitosan is derived from crustacean shells like crab and shrimp through deproteinization, demineralization, decolorization, and deacetylation. Its broad biological activities, antimicrobial, antifungal, antitumor, anticancer, anti-diabetic, antioxidant, and wound healing, make it highly valuable for drug delivery and biomedical applications,

including oral, nasal, ocular, pulmonary, mucosal, gene, and vaccine delivery, as well as tissue engineering. The scheme of synthesis of chitosan is depicted in Figure 9. (9, 49, 56, 58, 60)

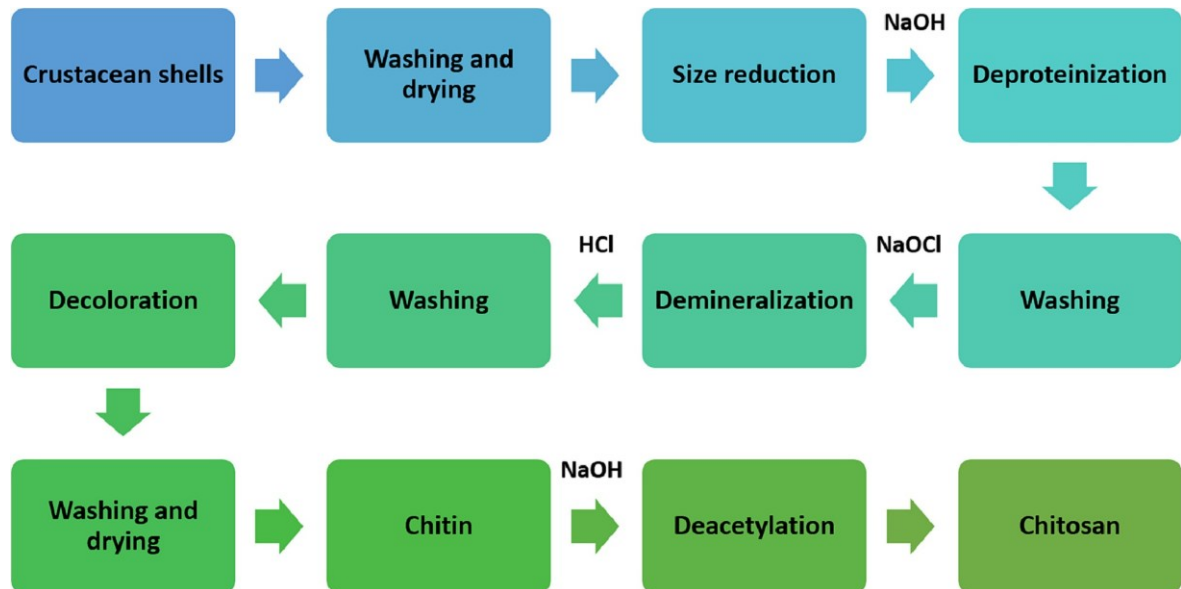


Figure 8 The scheme of synthesis of chitosan.

Source: (9)

3.5 Bioactive properties of chitosan

Chitosan exhibits a range of bioactive properties that make it attractive for biomedical and pharmaceutical applications. One of its key features is biokompatibility. Additionally, chitosan possesses antimicrobial activity against a broad spectrum of bacteria and fungi, primarily due to its polycationic nature, which disrupts microbial membranes. (9, 56)

Another important bioactivity of chitosan is its antioxidant capacity, enabling it to scavenge reactive oxygen species and reduce oxidative stress in biological environments. Chitosan has also been shown to exert immunomodulatory effects, including the stimulation or suppression of specific cytokine responses, depending on its molecular weight, degree of deacetylation, and formulation. These properties, combined with its biodegradability and ability to promote wound healing and tissue regeneration, underline chitosan's versatility as a bioactive material in modern biotechnological and therapeutic strategies. (9, 49, 56)

3.5.1 Mucoadhesion and permeation enhancement

With chitosan, mucoadhesion is primarily attributed to its cationic nature. The mucus gel layer contains anionic groups such as sialic acid and sulfonic acid, and the ionic interaction between chitosan's cationic groups and the anionic functionalities of the mucus facilitates mucoadhesion. The mucoadhesion of chitosan improves with an increase in molecular weight and the degree of deacetylation, although excessive cross-linking can reduce this property. This feature allows chitosan to effectively adhere to the upper respiratory and gastrointestinal tracts, promoting sustained drug release. (9, 56, 61)

While interacting with the mucus membrane, chitosan induces the opening of tight junctions between cells, which facilitates the passage of substances across the mucosal cells and enhances drug permeation. Therefore, chitosan (with a high molecular weight and a high degree of deacetylation) improves epithelial permeability. Modified forms of chitosan show greater permeation than unmodified chitosan, thiolation of chitosan further improves its permeation-enhancing properties on the mucosal membrane. (9, 61)

Chitosan can form ionic cross-links, leading to the creation of stable complexes that facilitate prolonged and controlled drug release. When combined with anionic polymers such as alginate, carrageenan, hyaluronic acid, pectin, and polyacrylates, chitosan forms stable complexes that enable the sustained release of drugs. (9, 49)

3.5.2 Biocompatibility and antimicrobial activity

Chitosan is highly biocompatible due to its structural and functional similarities to glycosaminoglycans, which are naturally present in the extracellular matrix of the body. (9, 56, 62)

Chitosan and its derivatives exhibit significant antimicrobial activity. The primary mechanism of action of chitosan's antimicrobial activity is attributed to its positive charge. Chitosan binds to bacterial DNA and interacts with surface molecules, leading to the inhibition of mRNA synthesis. (9, 62, 63)

Chitosan's antimicrobial activity is influenced by its molecular weight. High molecular weight chitosan acts extracellularly, preventing nutrient uptake and altering cell membrane permeability, while low molecular weight chitosan impacts both intracellular and extracellular processes, affecting mitochondrial function and protein and RNA synthesis. The antimicrobial effect also varies by microorganism type. In Gram-positive bacteria,

teichoic acid binds with chitosan, and in Gram-negative bacteria, lipopolysaccharide plays a similar role in disrupting the membrane. Higher concentrations of chitosan increase its positive charge, leading to bacterial agglutination. (9, 62, 63)

3.5.3 Antioxidant and anti-inflammatory activity

Oxidative stress can lead to various disorders, including inflammation, diabetes, and cancer. Chitosan and its derivatives protect against oxidative damage by scavenging free radicals and disrupting oxidation. Their antioxidant activity depends on the degree of deacetylation and molecular weight (MW), with low MW oligomers being more effective. Chitosan donates hydrogen atoms to halt oxidation, reducing malondialdehyde and fatty acid levels while boosting antioxidant enzymes. (9, 64, 65)

Inflammation is the body's response to injury, involving immune cells that aim to restore tissue integrity. Chitosan and its derivatives have been studied for their anti-inflammatory and pro-inflammatory effects. The anti-inflammatory action of chitosan is attributed to its conversion into glucosamine hydrochloride or salts like phosphate and sulfate, which are used to repair damaged tissues. Additionally, low molecular weight chitosan suppresses antigen-stimulated degranulation and cytokine production, showing potential in alleviating allergy-related inflammation and lung inflammation. (66, 67)

3.6 Chitooligosaccharides

Chitosan contains relatively unstable glycosidic bonds, making it prone to cleavage by hydrolyzing agents, which results in the production of chitosan oligomers with varying degrees of polymerization. Chitosans with a degree of polymerization of less than 20 and an average molecular weight of less than 3.9 kDa are referred to as chitooligosaccharides, chitosan oligomers, or chitooligomers. (10, 58, 59)

Chitooligosaccharides exhibit a wide range of biological activities and hold significant promise for applications in various fields, including medicine, cosmetics, food, and agriculture. Due to their low molecular weight and water solubility, they are in much higher demand compared to the precursor chitosan molecule. (10, 58, 59)

Homochitooligosaccharides are oligomers composed exclusively of either D-glucosamine (D unit) or N-acetyl-D-glucosamine (A unit), whereas heterochitooligosaccharides contain both D and A units. These hetero-oligomers vary in degree of polymerization, degree of acetylation, degree of deacetylation, and the position of

N-acetyl residues in the oligomer chain. Heterochitooligosaccharides with a degree of polymerization less than 10 are typically water-soluble, but the solubility of those with a degree of polymerization greater than 10 depends on their degree of acetylation and the pH of the solution. Different chitooligosaccharides may share the same degree of polymerization but differ in their fraction of N-acetylated residues. (10, 58, 59)

Research has shown that chitooligosaccharides can prevent tumor growth, treat asthma, improve bone strength, prevent malaria, and serve as vectors in gene therapy. The biological activities include antibacterial, antifungal, antiviral, anti-tumor, antioxidant, immunoregulatory effects, and the ability to control fat levels, blood pressure, and cholesterol. Chitooligosaccharides with a degree of polymerization longer than hexamers have been shown to possess greater bioactivity, including enhanced antimicrobial, anti-tumor, and immunopotential effects, compared to smaller chitooligosaccharides. Although numerous studies have demonstrated the biological activities of chitooligosaccharides, the molecular mechanisms behind these effects are not yet fully understood. (10, 59, 67)

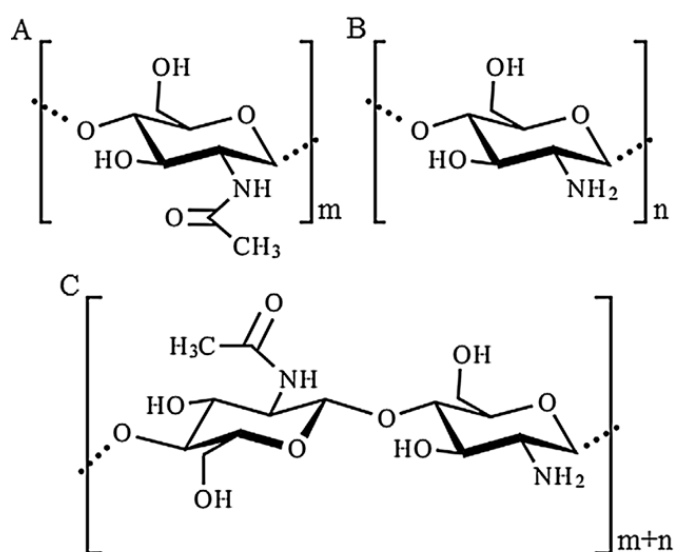


Figure 9 Structure of N-acetylated chitooligosaccharide (A), N-deacetylated chitooligosaccharide (B) and partially N-deacetylated chitooligosaccharide, $m/n/m+n$ =degree of polymerization=2-20.

Source: (10)

II. PRACTICAL PART

4 PREPARATION OF COLLOIDAL PARTICLES

The purpose of this analysis was to prepare colloidal particles combining polypyrrole as a conductive polymer with four types of chitooligosaccharides. First, chitosan was depolymerized, and the resulting chitooligosaccharides were used for the synthesis of colloidal particles. The material was characterized by evaluating particle size and size distribution, measuring zeta potential, and assessing biocompatibility and potential immunomodulatory properties *in vitro* using adherent cell lines of murine fibroblasts (NIH/3T3) and murine macrophages (RAW 264.7).

4.1 Materials and equipment

- Chitosan, low molecular weight (Sigma Aldrich Co., USA)
- Acetic acid glacial (VWR Chemicals, USA)
- Ultrapure water (UPW)
- Nitrogen gas (N₂)
- Sodium nitrite (NaNO₂, Thermo Fisher Scientific, USA)
- Hydrochloric acid (HCl, 37%, Penta, Czech Republic)
- Deacetylated chitosan (DA approx. 0%, synthesized by the research workers of the Centre of Polymers in Zlín, Czech Republic)
- Pyrrole (99%, Thermo Fischer Scientific, USA)
- Sodium chloride (NaCl, Merck & Co., USA)
- Mouse embryonic fibroblast cell line (NIH/3T3, ECACC 93061524, England)
- Mouse macrophage cell line (RAW 264.7, ATCC-TIB-71, Germany)
- Dulbecco's Phosphate Buffered Saline (PBS, pH 7.2, Biosera, France)
- Trypsin (Biosera, France)
- Dulbecco's Modified Eagle's Medium with Phenol Red (DMEM, high glucose, Biosera, France)
- DMEM without Phenol Red (high glucose, Capricorn Scientific, Germany)
- Fetal Bovine Serum (FBS, Biowest, South America)

- Sodium Pyruvate (Gibco, USA)
- Lipopolysaccharides from *Escherichia coli* 026:B6 (LPS, Sigma Aldrich Co., USA)
- Thiazolyl Blue Tetrazolium Bromide (MTT, Duchefa Biochemie, The Netherlands)
- Griess reagent (Sigma Aldrich Co. USA)
- Dimethylsulfoxide (DMSO, Duchefa Biochemie, The Netherlands)
- Mouse IL-6 Uncoated ELISA Kit (Thermo Fisher Scientific, USA)
- Sulfuric acid (H₂SO₄, 95-98%, Sigma Aldrich Co. USA)
- Tween 20 (Carl Roth GmbH and Co., Germany)
- Basic laboratory glassware (beakers, pipettes, boiling flasks, reagent bottles)
- Micropipettes (Eppendorf, Germany)
- Magnetic stirrer (Heidolph Instruments GmbH and Co., Germany) and magnetic bars
- Syringe Filters (PES, 0.22 µm and 0.45 µm, VWR Chemicals, USA) and syringes (B. Braun Medical Inc., Germany)
- Dialysis Membrane MWCO 100-500 Da (Spectra/Por®, USA)
- Centrifugal Filters (100 kDa, Thermo Fischer Scientific, USA)
- Laminar flow hood Bio130 A2 (Alpina, Poland)
- Freeze Dryer (LaboGene, Denmark)
- Zetasizer Nano (Malvern Panalytical, United Kingdom)
- (PerkinElmer, USA)
- Tissue culture test plates, tubes and microtubes, cell scrapers
- Incubator Heracell™ 150i (Thermo Fischer Scientific, USA)
- Centrifuge 5702 R (Eppendorf, Germany)
- Microcentrifuge MiniSpin (Eppendorf, Germany)
- Vortex mixer (VELP Scientifica Srl, Italy)
- Inverted microscope IX51 (Olympus Co., Japan)
- Photometer Infinite 200 Pro M Nano (Tecan Group Ltd., Switzerland)

4.2 Depolymerization of chitosan

Chitosan samples were dissolved in 2.5% (v/v) acetic acid at a concentration of 20 mg/mL using a magnetic stirrer for 24 hours (the chitooligosaccharides OCH1 and OCH3 were obtained from commercial chitosan, while samples OCH5 and OCH6 were prepared from deacetylated chitosan, see Chapter 4.1). Subsequently, three additions of sodium nitrite (NaNO_2) were carried out at 45-minute intervals. Prior to each addition, ultrapure water (UPW) was purged with a continuous flow of nitrogen gas for 15 minutes and then used to prepare a fresh NaNO_2 solution at a concentration of 20 mg/mL. Each NaNO_2 solution was prepared immediately before use, in separate batches for each chitosan sample.

Before each NaNO_2 addition, the chitosan solutions were also purged with nitrogen gas for 15 minutes (Figure 10) to ensure inert conditions for the reaction. Between additions, both the chitosan samples and the UPW were stored in a refrigerator, with the samples continuously stirred throughout the process. The total volume of NaNO_2 solution added to each sample was based on the desired chitooligosaccharide chain length, and was provided by researchers at the Centre of Polymers in Zlín from previous experiments.

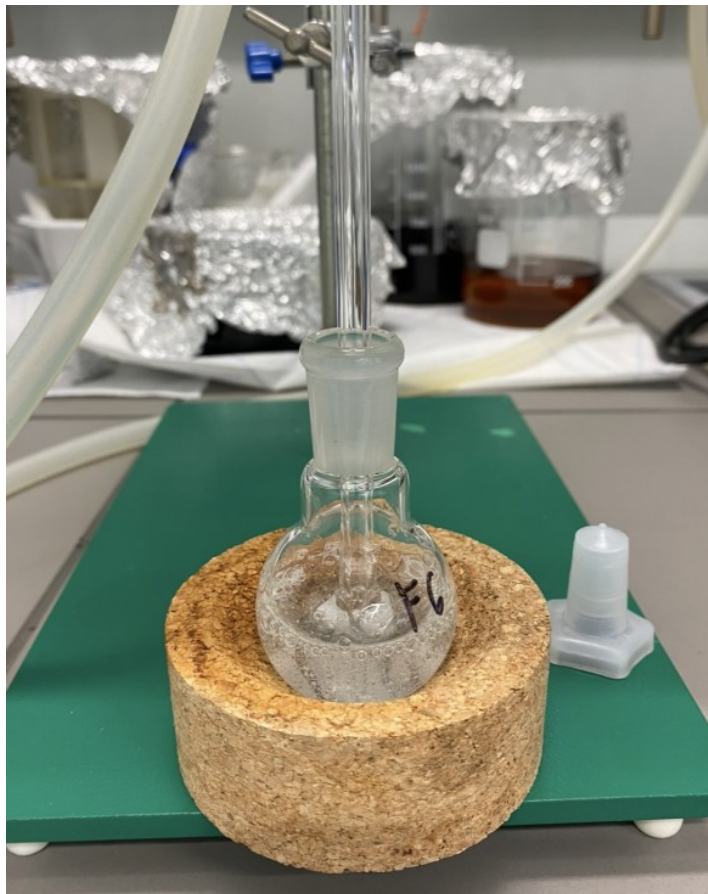


Figure 10 Purging of sample OCH6 with nitrogen gas prior to the addition of NaNO_2 .

Following the final addition, the samples were left in the refrigerator overnight under constant stirring to complete the depolymerization. The next day, they were filtered through a 0.22 μm syringe filter into dialysis membranes with a molecular weight cut-off of 100–500 Da, which had been pre-soaked in UPW overnight. Dialysis was performed in 2 L of UPW, with the water replaced halfway through the process (Figure 11).

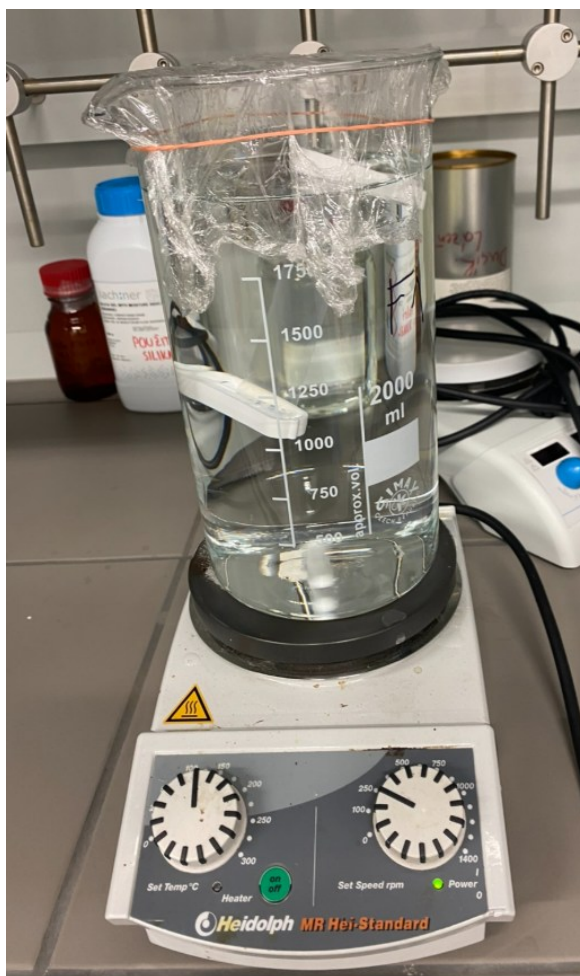


Figure 11 Dialysis of sample OCH1 in ultrapure water using a membrane with a molecular weight cut-off of 100-500 Da.

The duration of dialysis and the corresponding volume of NaNO_2 solution for each sample, along with the resulting chitooligosaccharide chain lengths and molecular weights, are summarized in Table 2. The molecular weights were provided by researchers at the Centre of Polymers in Zlín and were determined using proton nuclear magnetic resonance (^1H NMR) spectroscopy.

The samples were then freeze-dried for 72 hours. After dialysis, the solutions were filtered again and carefully transferred drop by drop into beakers pre-cooled in liquid nitrogen. The beakers were covered with precision wipes and placed in the freeze dryer to

complete the lyophilization process. The dried samples were then transferred into tubes and stored at $-20\text{ }^{\circ}\text{C}$.

Table 2 Sample name, chain length, total volume of added NaNO_2 , dialysis duration and molecular weight of all chitooligosaccharides.

Sample name	Chain length	Total volume of NaNO_2 solution [mL]	Dialysis duration [hrs]	Molecular weight [g/mol]
OCH9	Nonamer	1.000	4	1479.933
OCH8	Octamer	1.393	2	1289.267
OCH5	Pentamer	1.960	2	769.681
OCH2	Dimer	5.545	2	323.295

4.3 Synthesis of colloidal particles

Samples prepared in Chapter 4.2 were dissolved in 0.01M HCl in a concentration of 10 mg/mL. Subsequently, pyrrole was added in 0.1M abundance. The reaction mixture was placed in a water bath and was left reacting overnight at $50\text{ }^{\circ}\text{C}$ while being stirred constantly (Figures 10 and 11). From this point onward, the samples are labeled with an underscore (_ppy) to indicate the addition of polypyrrole.

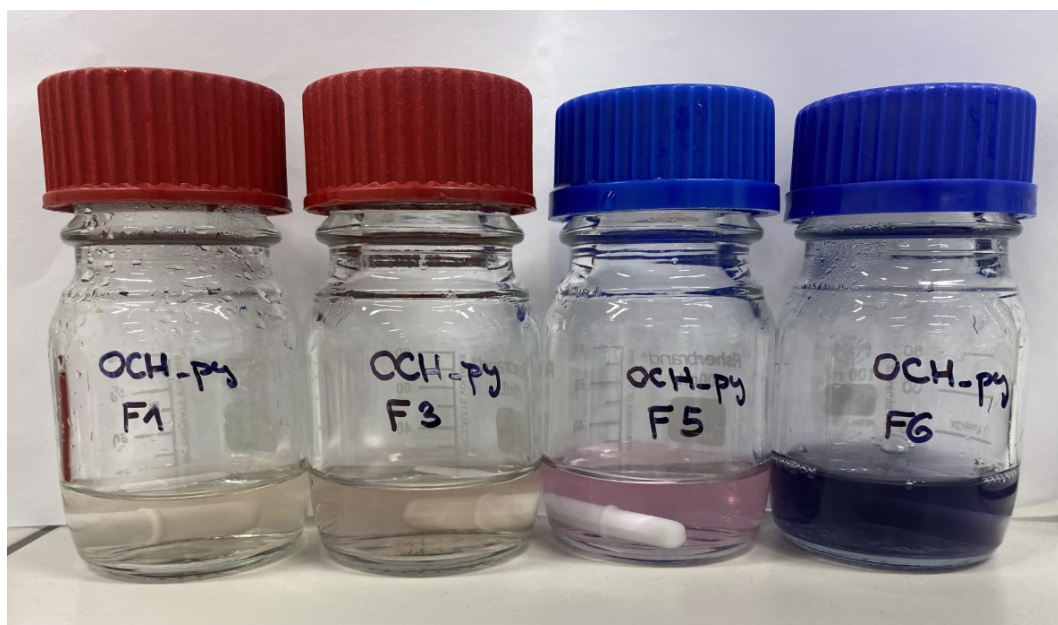


Figure 12 Reaction mixture five minutes after the start of the reaction. The samples from left to right: OCH9_ppy, OCH5_ppy, OCH8_ppy, OCH2_ppy.

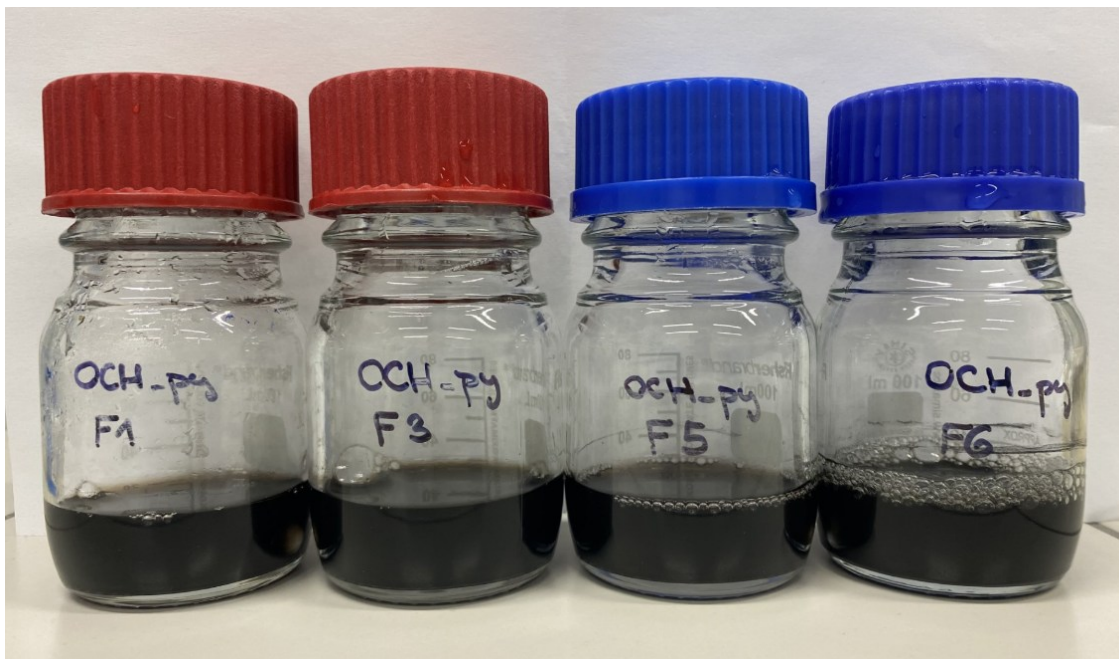


Figure 13 Reaction mixture ten minutes after the start of the reaction. The samples from left to right: OCH9_ppy, OCH5_ppy, OCH8_ppy, OCH2_ppy.

The next day, the colloids were transferred into a dialysis membrane with a molecular weight cut-off of 100–500 Da and dialyzed for one day in 0.01 M NaCl, followed by three days in deionized water. The dialysis was performed in a laminar flow hood. Subsequently, the colloids were ultracentrifuged using tubes equipped with ultracentrifugal filter units with a 100 kDa cut-off and then stored in a refrigerator.

4.4 Characterization of colloidal particles

The concentration of the prepared colloids was determined using gravimetric analysis. Empty microtubes were carefully labeled and weighed using an analytical balance scale. Subsequently, 250 μL of each colloid sample was transferred into the labeled microtubes and dried in an oven at 70 $^{\circ}\text{C}$ for 24 hours. The dried residues were then weighed, and the concentration per 250 μL was calculated based on the difference between the initial and final masses.

Particle size, size distribution and zeta potential were measured using a Zetasizer Nano. The samples were diluted with UPW to a concentration of 0.25 mg/mL, filtered through a 0.45 μm syringe filter, and transferred into a folded capillary cuvette. The cuvette was placed into the measuring cell. The measurement settings included an equilibration time of 60 seconds, a minimum run count of 25, a maximum run count of 100, and five measurements performed per sample.

The UV/VIS absorbance spectra of the colloidal particle suspensions were recorded using a UV/VIS spectrophotometer. Prior to measurement, the samples were diluted appropriately with UPW to prevent saturation of the detector. Samples OCH1_ppy, OCH3_ppy, and OCH5_ppy were diluted to a concentration of 0.25 mg/mL. Sample OCH6_ppy was diluted to 1.25 mg/mL, as it contained the highest amount of polypyrrole (being a dimer), which gave it a more intense black tint compared to the other samples. Measurements were carried out in quartz cuvettes with a 1 cm path length. A baseline correction was performed using UPW as a blank. Absorbance spectra were recorded in the wavelength range of 200–800 nm, and characteristic absorbance peaks were analyzed to assess the presence of a conjugated system to confirm colloid formation.

4.5 Biocompatibility and bioactivity evaluation

To assess the potential biomedical applicability of the synthesized colloidal particles, both biocompatibility and bioactivity were evaluated using *in vitro* assays. Biocompatibility was determined using the MTT assay on NIH/3T3 mouse fibroblasts and RAW 264.7 mouse macrophages to evaluate cell viability in response to the colloidal formulations. Bioactivity was assessed through nitric oxide (NO) production using the Griess reagent and the quantification of the pro-inflammatory cytokine interleukin-6 (IL-6) via ELISA, both performed on the RAW 264.7 macrophage cell line. These assays provide insight into the cytotoxicity and potential immunomodulatory properties of the particles.

4.5.1 Preparation of complete DMEM

For the NIH/3T3 cell line, powdered DMEM (with Phenol Red) was used to prepare the base medium. The powder was dissolved in ultrapure water according to the manufacturer's instructions to achieve a final volume of 1 L. Fetal bovine serum was added in a concentration of 10% (v/v). The solution was then sterile-filtered through a 0.22 μ m membrane (Figure 14), supplemented with 1% Penicillin/Streptomycin and mixed thoroughly.

For the RAW 264.7 cell line, the medium was prepared using the same steps but was supplemented further with 1% sodium pyruvate (v/v) and 2mM L-glutamine. Medium containing Phenol Red was used for the MTT assay. For the NO production assay and ELISA, cells were cultured in medium without Phenol Red but supplemented identically.

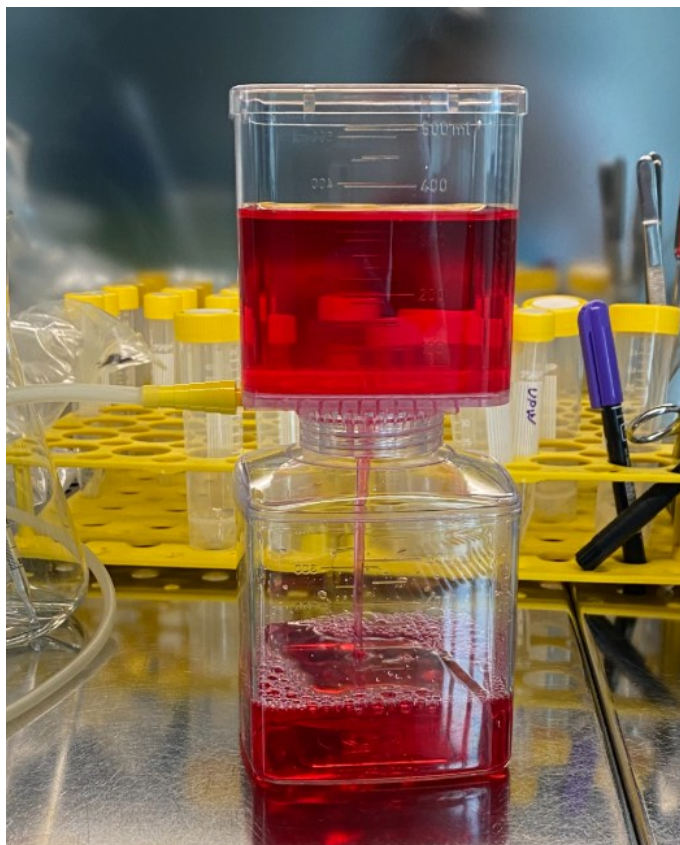


Figure 14 Sterile filtration of DMEM supplemented with fetal bovine serum (FBS) using a 0.22 µm membrane.

4.5.2 MTT assay

To evaluate the cytotoxicity of the prepared colloidal particles, an MTT assay was performed using NIH/3T3 mouse fibroblasts and RAW 264.7 mouse macrophages.

NIH/3T3 cells for cytocompatibility evaluation were prepared by discarding the culture medium from the flask, rinsing with PBS and trypsinizing. The same amount of DMEM as trypsin was added, transferred into a test tube and placed in a centrifuge at 3 min/1100 RPM. The supernatant was then removed. The cell pellet was resuspended in the culture medium and diluted to the concentration needed.

RAW 264.7 cells were cultured on a Petri dish. For the MTT assay, the culture medium was discarded and 2 mL of fresh medium was added into the dish. The cells were gently scraped with a cell scraper, homogenized by repetitive pippeting and diluted as needed.

Cells were seeded into 24-well plates at a concentration of 250×10^3 cells/well and incubated at 37 °C with 5% CO₂ for 24 hours to allow attachment. Afterwards, the medium was replaced with 500 µL of fresh medium containing colloidal particles at various

concentrations. The macrophages were treated with lipopolysaccharide (LPS) at a concentration of 25 ng/mL in combination with the colloidal particles, serving both as a positive control and to stimulate an immune response. Cells were incubated for an additional 24 hours under the same conditions.

Following the treatment, the medium with samples was discarded and 500 μ L of MTT reagent (5 mg/mL in UPW, 9:1 medium:MTT solution) was added to each well and incubated for 6 hours at 37 °C. The resulting formazan crystals were solubilized by carefully removing the medium and adding 500 μ L of DMSO to each well. Absorbance was measured at 570 nm using a microplate reader. The values of 690 nm were subtracted from those of 570 nm. Cell viability was expressed as a percentage relative to untreated control cells (NIH/3T3) or cells treated with LPS only (RAW 264.7).

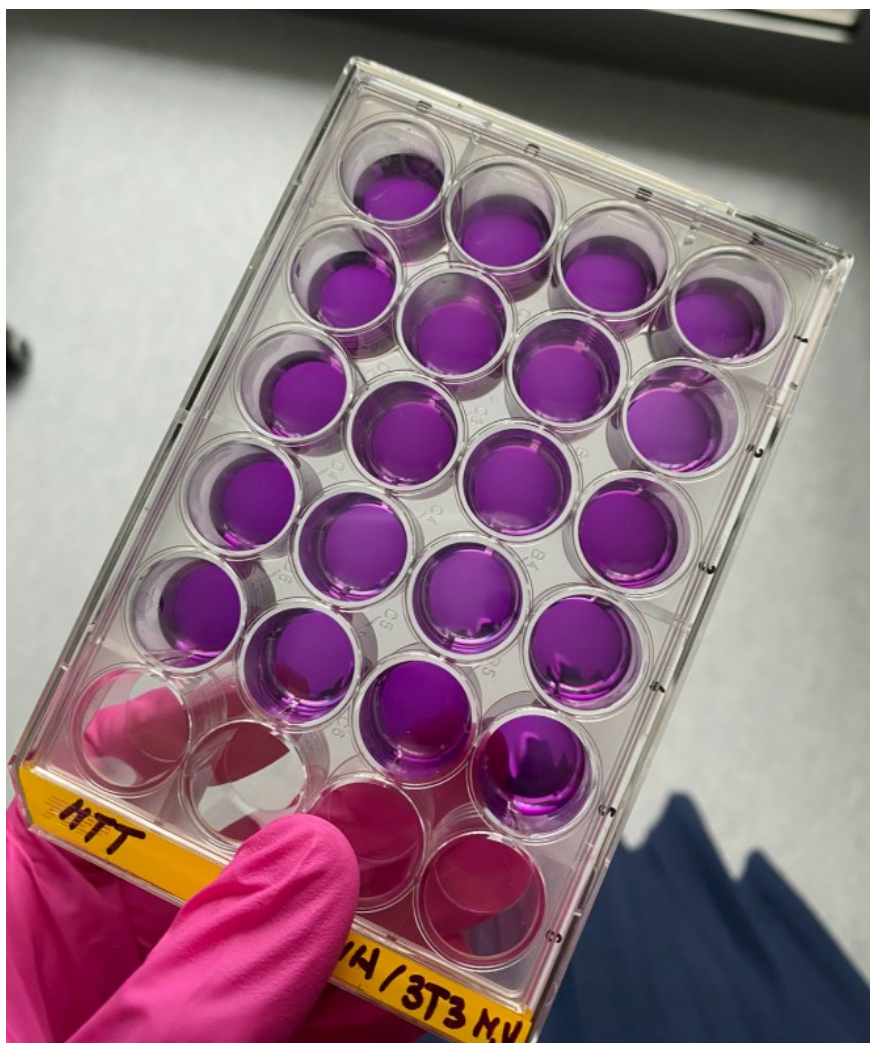


Figure 15 Tissue culture plate with NIH/3T3 cell line after dissolution of formazan crystals in DMSO. Samples OCH9_ppy and OCH8_ppy were used in this assay.

4.5.3 NO production (RAW 264.7)

To assess NO production as an indicator of macrophage activation, RAW 264.7 cells were diluted as in Chapter 4.5.2, seeded in 24-well plates at a density of 250×10^3 cells/well and incubated for 24 hours at 37 °C in 5% CO₂. After adherence, the cells were treated with colloidal samples diluted in DMEM without Phenol Red and LPS (25 ng/mL) and incubated for an additional 24 hours.

After incubation, the medium from each well was transferred into microtubes and centrifuged using a microcentrifuge to eliminate potential interference from residual colloids in the subsequent analysis. Then, 50 µL of cell culture supernatants were transferred to a new 96-well plate, and 50 µL of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride) was added. The plate was incubated at room temperature for 10 minutes, and absorbance was measured at 540 nm. Nitrite production was expressed as a percentage relative to cells treated with only LPS.

4.5.4 IL-6 production (RAW 264.7)

IL-6 production was measured using RAW 264.7 macrophages with a mouse IL-6 ELISA kit according to the manufacturer's instructions. Cells were seeded at a density of 250×10^3 cells/well in a 24-well plates and incubated overnight. The medium was then replaced with DMEM containing colloidal samples at various concentrations and LPS. Cells were then incubated for 24 hours.

After treatment, the culture supernatants were collected and used for IL-6 quantification. The ELISA kit was used according to the manufacturer's instructions. First, a 96-well plate was coated with the capture antibody, incubated overnight and washed. Standards and samples were incubated with the detection antibody. After washing and addition of the enzyme conjugate and substrate solution, the reaction was stopped with a stop solution (2N H₂SO₄). Between each step, the plate was washed with a wash buffer (0.05% Tween 20 in PBS).

Absorbance was measured at 450 nm with a reference wavelength of 570 nm using a microplate reader, IL-6 secretion was expressed relevant to a positive control of cells treated with LPS only.

5 RESULTS AND DISCUSSION

5.1 Characterization of colloidal particles

Colloidal particles combining polypyrrole with four different lengths of chitooligosaccharide chains were prepared. The synthesis was carried out without the use of oxidizing agents, with the aim of aligning the process with the principles of green chemistry and enhancing the potential biocompatibility of the final material. Instead of an oxidizing agent, the functional groups of the biopolymers acted as both stabilizers and catalysts for the formation of polypyrrole, potentially resulting in stable colloidal particles.

The first characterization method used was gravimetric analysis to determine the concentration of particles in the colloidal system. Table 3 lists the masses of the empty microtubes and the masses measured after water evaporation. Outlying data points were excluded using Grubbs' test for outliers at a significance level of $\alpha = 0.05$.

Table 3 Masses of empty microtubes, masses after water evaporation, and the calculated differences for each sample, along with their averages. Concentrations were calculated excluding outlying values, which are marked with an asterisk (*).

Sample	Replicate	Empty [mg]	After drying [mg]	Difference [mg]	Average [mg]	Concentration [mg/mL]
OCH9_ppy	1	1086.45	1088.47	2.02	1.96 ± 0.12	7.86
	2	1073.26	1075.39	2.13		
	3	1082.69	1084.62	1.93		
	4	1072.86	1074.74	1.88		
	5	1087.12	1088.98	1.86		
OCH8_ppy	1	1086.11	1087.40	1.29	1.27 ± 0.09	5.10
	2	1082.00	1083.26	1.26		
	3	1084.03	1085.23	1.20		
	4	1088.21	1089.40	1.19		
	5	1087.40	1088.83	1.43		
OCH5_ppy	1	1084.03	1084.93	0.90	0.93 ± 0.04	3.72
	2	1086.84	1087.74	0.90		
	3	1080.03	1081.27	1.24*		
	4	1088.03	1088.99	0.96		
	5	1088.55	1089.51	0.96		
OCH2_ppy	1	1088.58	1088.82	0.24	0.34 ± 0.09	1.36
	2	1085.80	1086.20	0.40		
	3	1086.97	1087.25	0.28		
	4	1084.31	1084.88	0.57*		
	5	1085.60	1086.04	0.44		

Using the Zetasizer Nano, the hydrodynamic diameter and zeta potential were measured. Table 4 lists the results from particle size and distribution obtained for each sample. The data presented in are arithmetic averages of five replicate measurements of particle size and distribution, with outliers excluded using Grubbs' test at a significance level of $\alpha = 0.05$.

Table 4 Hydrodynamic diameter (d) values and polydispersity index (PdI) for each sample. Up to three peaks were observed in each measurement, therefore the mean and area of each peak are included.

Sample	d [d·nm]	PdI	Peak Mean [d·nm]			Peak Area [%]		
OCH9_ppy	126.5	0.5	240.5	3.0	0.0	99.8	0.2	0.0
OCH8_ppy	139.0	0.4	241.3	3.6	0.0	97.4	2.6	0.0
OCH5_ppy	62.4	1.0	871.4	4.8	2.1	71.1	21.0	8.0
OCH2_ppy	60.6	1.0	428.6	4.7	0.5	78.9	16.1	5.0

The hydrodynamic diameters of the primary particle populations ranged from 60.6 nm (OCH2_ppy) to 139.0 nm (OCH8_ppy). The smallest particles were observed in OCH2_ppy, which was synthesized using the shortest chitooligosaccharide chain (dimer). This suggests that shorter biopolymer chains may allow for more compact polypyrrole structures or more efficient stabilization of smaller colloidal species.

In contrast, OCH9_ppy (nonamer) and OCH8_ppy (octamer) formed slightly larger particles, with diameters of 126.5 nm and 139.0 nm, respectively. The size increase in these samples could be attributed to steric effects introduced by longer chain lengths, which may result in less efficient stabilization during pyrrole polymerization.

OCH5_ppy (pentamer) showed a different behavior, with a significant second peak centered at 871.4 nm, accounting for 21% of the total intensity, indicating the presence of large aggregates or loosely associated clusters. This sample also had a high PdI value (1.0), reflecting high polydispersity and a broad particle size distribution. This may suggest less effective stabilization or heterogeneous polypyrrole deposition under the specific conditions used.

The PdI values support these observations. OCH8_ppy and OCH9_ppy showed intermediate PdI values (0.4 and 0.5), indicating moderately monodisperse systems. OCH2_ppy, despite its small size, exhibited a high PdI (1.0), which may result from a mixture of individual particles and small aggregates, or from inconsistencies due to its more intense black tint compared to the other samples. Nonetheless, the main peak (78.9%)

remained dominant, suggesting that the colloid was still largely composed of nanoscale particles.

In all samples, the primary peaks accounted for more than 70% of the total area, indicating that the majority of particles formed were within the nanometer size range. However, the presence of secondary peaks in OCH5_ppy and OCH2_ppy suggests that aggregation may occur to a limited extent, especially in systems with shorter or more flexible chains.

Overall, the DLS results show a relationship between chitooligosaccharide chain length, particle size, and polydispersity, with shorter chains promoting smaller particle formation but potentially increasing the risk of instability or aggregation. Longer chains appear to produce larger, more stable particles, likely due to more effective steric stabilization during polypyrrole polymerization.

In addition to hydrodynamic diameter and zeta potential, the conductivity of each colloidal sample was recorded. Conductivity is a relevant parameter in zeta potential analysis, as it reflects the ionic strength of the dispersion medium, which influences the thickness of the electrical double layer surrounding the particles. Monitoring conductivity ensures consistent measurement conditions and allows for more accurate interpretation of colloidal stability. The values obtained helped confirm that the differences in zeta potential were not due to uncontrolled variations in the medium's ionic environment. The data presented in Table 5 are arithmetic averages of five replicate measurements of zeta potential and conductivity, with outliers excluded using Grubbs' test at a significance level of $\alpha = 0.05$.

Table 5 Zeta potential and conductivity values for each sample

Sample	Zeta potential [mV]	Conductivity [mS/cm]
OCH9_ppy	15.4 ± 0.3	0.0556 ± 0.0012
OCH8_ppy	13.93 ± 0.07	0.0582 ± 0.0019
OCH5_ppy	0.53 ± 0.05	0.0742 ± 0.0006
OCH2_ppy	-0.18 ± 0.03	0.1785 ± 0.0018

The zeta potential values reveal a clear trend regarding the influence of chitooligosaccharide chain length on particle surface charge. OCH9_ppy (nonamer) and OCH8_ppy (octamer) exhibited the highest positive zeta potentials: 15.4 ± 0.3 mV and 13.93 ± 0.07 mV, respectively. These values indicate moderate electrostatic stability and suggest effective

surface coverage by the longer biopolymer chains, which carry positively charged amino groups. In contrast, OCH5_ppy (pentamer) showed a significantly lower zeta potential of 0.53 ± 0.05 mV, and OCH2_ppy (dimer) even exhibited a slightly negative zeta potential (-0.18 ± 0.03 mV). These results indicate minimal surface charge, which correlates with the high polydispersity ($PdI = 1.0$) and evidence of aggregation observed in DLS data. The reduced electrostatic repulsion likely facilitates particle clustering in these samples.

The conductivity measurements further support these observations. OCH2_ppy, the least stable sample with the lowest zeta potential, displayed the highest conductivity (0.1785 ± 0.0018 mS/cm). This suggests a greater concentration of mobile ions in the dispersion, potentially due to weak surface binding of counterions or dissociation from aggregated or loosely stabilized particles. OCH9_ppy and OCH8_ppy had lower conductivity values (0.0556 and 0.0582 mS/cm, respectively), consistent with more stable colloidal systems where ionic species are more strongly associated with the particle surface. OCH5_ppy showed intermediate conductivity (0.0742 ± 0.0006 mS/cm), which corresponds with its marginally positive zeta potential and partial aggregation behavior in DLS.

These findings align with the hypothesis that longer chitooligosaccharide chains provide better electrostatic stabilization, while shorter chains may fail to effectively shield or stabilize the polypyrrole particles.

The UV/VIS absorbance spectra of the colloidal particle suspensions were recorded using a UV/VIS spectrophotometer.

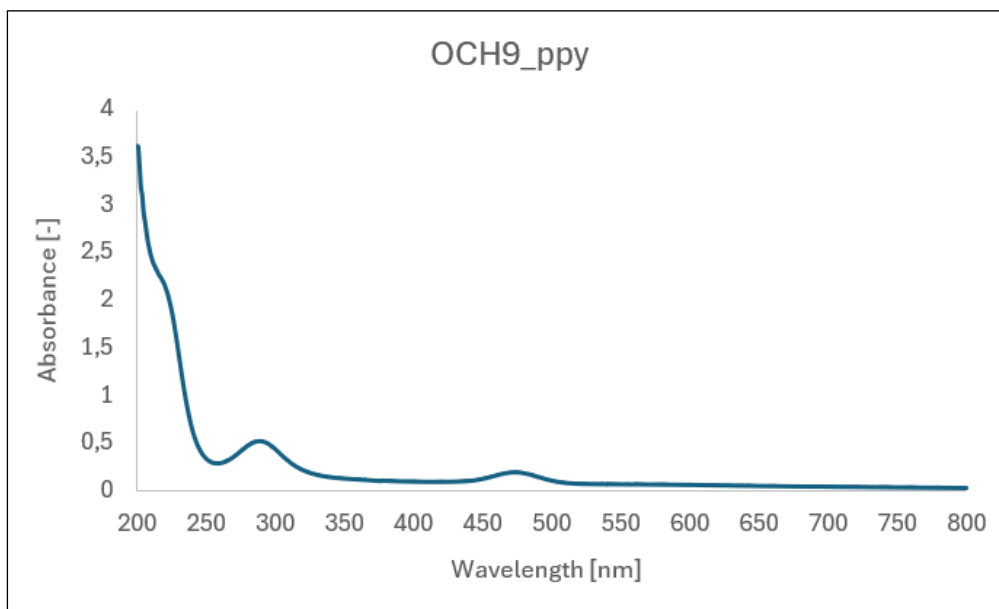


Figure 16 UV/VIS spectrum of sample OCH9_ppy measured at a concentration of 0.25 mg/mL. The spectrum features a pronounced absorption peak at approximately 290 nm and a smaller, broad peak near 475–485 nm.

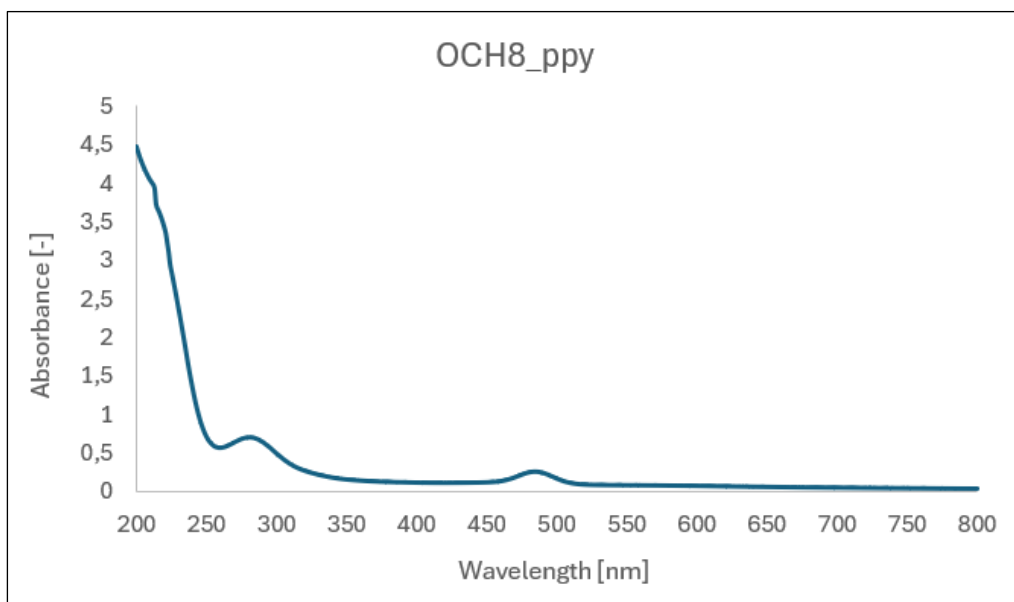


Figure 17 UV/VIS spectrum of sample OCH8_ppy measured at a concentration of 0.25 mg/mL. The spectrum shows a distinct peak near 290 nm and another visible peak around 480 nm.

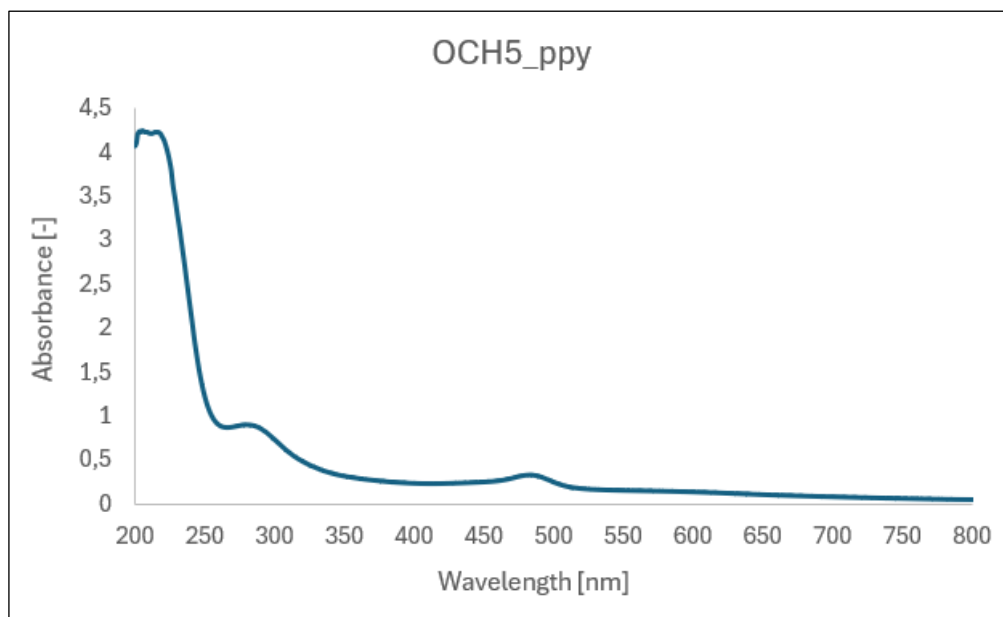


Figure 18 UV/VIS spectrum of sample OCH5_ppy measured at a concentration of 0.25 mg/mL. A strong absorbance maximum is observed at approximately 290 nm, with a secondary, less intense peak appearing around 475 nm.

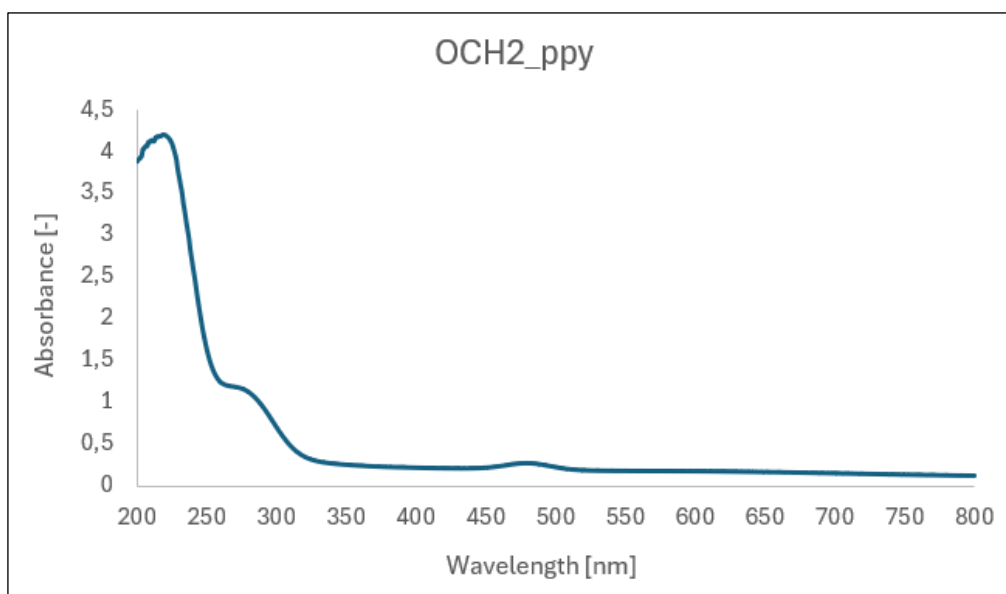


Figure 19 UV/VIS spectrum of sample OCH2_ppy measured at a concentration of 0.125 mg/mL. The spectrum displays a strong absorption band near 290 nm and a smaller, smooth peak centered around 480 nm.

UV/VIS spectroscopy was used to evaluate the optical properties of the colloidal particles synthesized from polypyrrole and chitooligosaccharides of varying chain lengths. All samples exhibited a prominent absorption peak near 290 nm, corresponding to π - π^* transitions within the conjugated polypyrrole backbone. This consistent feature across samples confirms successful incorporation of polypyrrole into the colloidal systems.

A smaller, broader absorption band appeared around 475–485 nm, which is typically associated with polaron or bipolaron states in doped polypyrrole. The presence and intensity of this band reflect differences in the oxidation state, chain alignment, and interaction of polypyrrole with the surrounding biopolymer matrix. (12, 68, 69)

The spectra also reflect the influence of chitooligosaccharide chain length and sample concentration. OCH9_ppy and OCH5_ppy, both measured at 0.25 mg/mL, showed relatively defined peaks at 290 nm with moderate shoulders at 480 nm. OCH8_ppy, also measured at 0.25 mg/mL, exhibited a broader absorption profile with a more noticeable secondary peak near 480 nm, suggesting a slightly higher degree of polypyrrole conjugation or interaction with the biopolymer. OCH2_ppy, measured at 0.125 mg/mL, displayed the strongest relative absorbance at 290 nm despite its lower concentration. This sample contained the highest proportion of polypyrrole per unit of biopolymer, leading to an intense black coloration and more prominent absorbance features. The peak at 480 nm was also defined, indicating electronic delocalization in the polypyrrole chains.

These findings suggest that shorter chitooligosaccharide chains, such as those in OCH2_ppy (dimer), may facilitate more efficient polypyrrole formation or better electronic interaction, possibly due to their lower steric hindrance and greater mobility during polymerization. Conversely, longer chains, such as in OCH9_ppy, may restrict polypyrrole growth or alignment, resulting in slightly less pronounced electronic features.

Overall, the UV/VIS spectra indicate that chitooligosaccharide chain length influences the optical properties and degree of conjugation of the resulting colloidal particles.

5.2 Biocompatibility and bioactivity evaluation

Firstly, the MTT assay was performed to assess the cytocompatibility of each sample. The assay was conducted using two murine cell lines: NIH/3T3 fibroblasts and RAW 264.7 macrophages. Fibroblasts were selected due to the potential application of the prepared conductive colloidal particles in wound healing, where restoration of the transepithelial potential plays an important role, as discussed in Chapter 2.6.2. NIH/3T3 is a commonly used model to assess wound healing and tissue regeneration potential. Macrophages were employed in all biocompatibility and bioactivity assays to evaluate the immunomodulatory effects of the particles, particularly their influence on nitrite production and interleukin-6 (IL-6) secretion. Figures 20–23 present the percentage of cell viability for NIH/3T3 fibroblasts, expressed relative to untreated control cells. Figures 24–27 show cell viability

for RAW 264.7 macrophages, expressed relative to cells treated with LPS alone, which served as the experimental control for immunological activation. An additional control consisting of completely untreated macrophages was assessed to verify the baseline viability of the cell passages used, however, these results are not included in the data discussed in this thesis, as they served solely as a quality control checkpoint. Outliers excluded using Grubbs' test at a significance level of $\alpha = 0.05$.

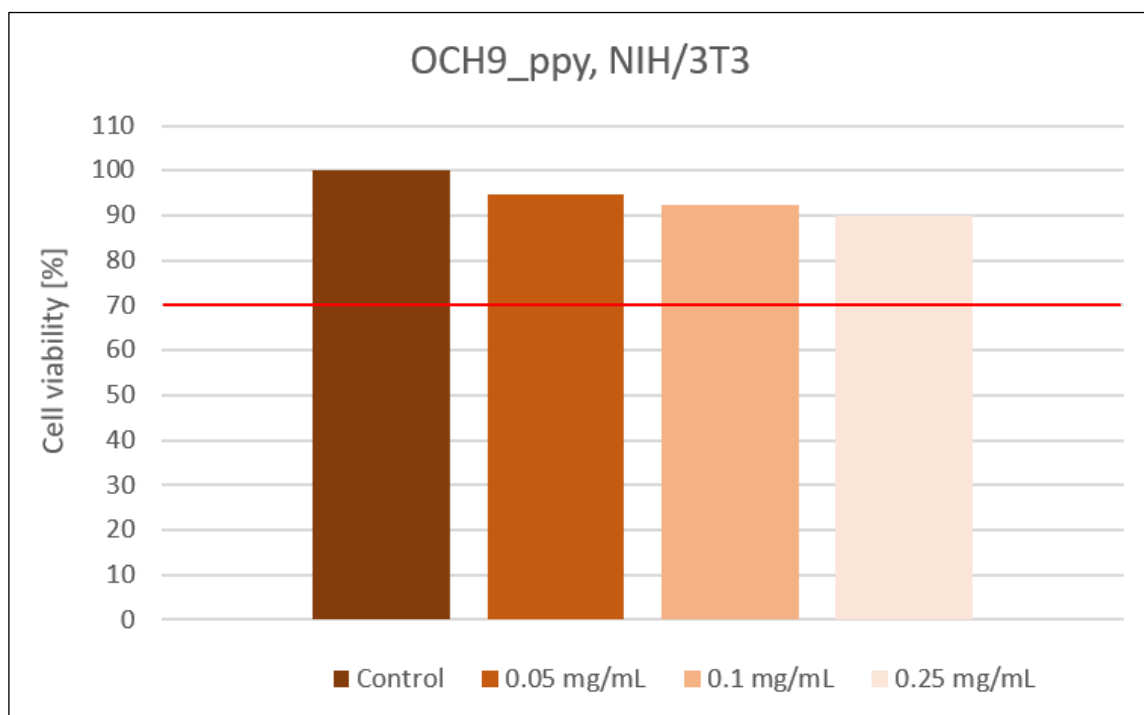


Figure 20 Cell viability of NIH/3T3 fibroblasts treated with OCH9_ppy sample, expressed relative to untreated control cells. The red line indicates the cytotoxicity threshold at 70% viability.

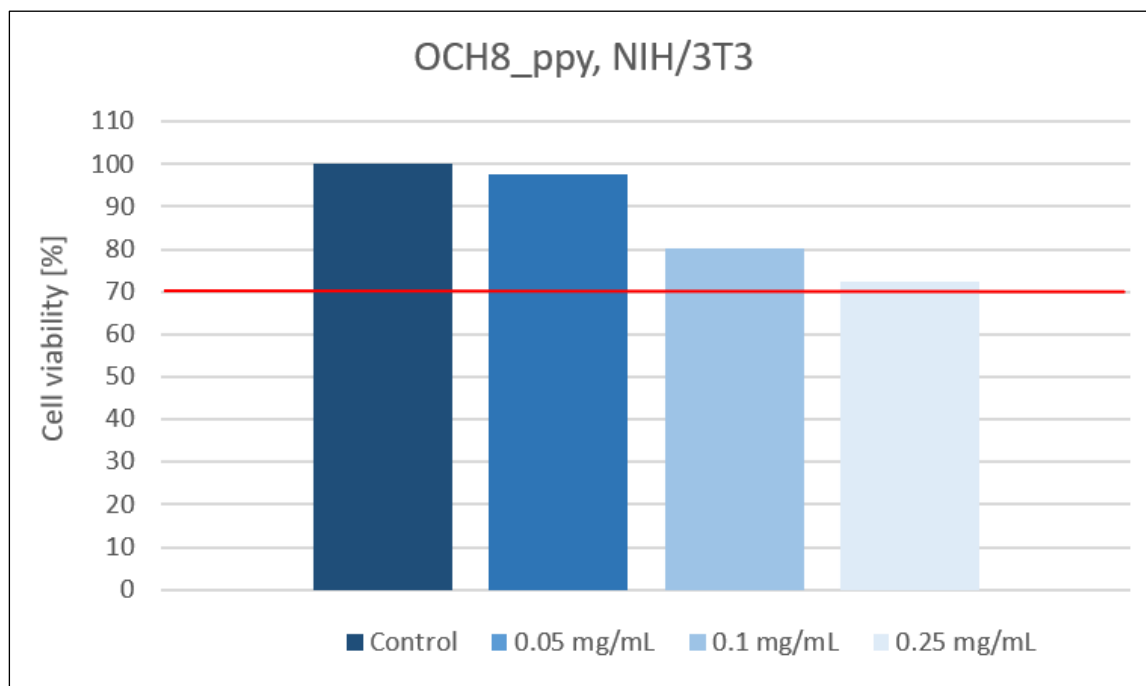


Figure 21 Cell viability of NIH/3T3 fibroblasts treated with OCH8_ppy sample, expressed relative to untreated control cells. The red line indicates the cytotoxicity threshold at 70% viability.

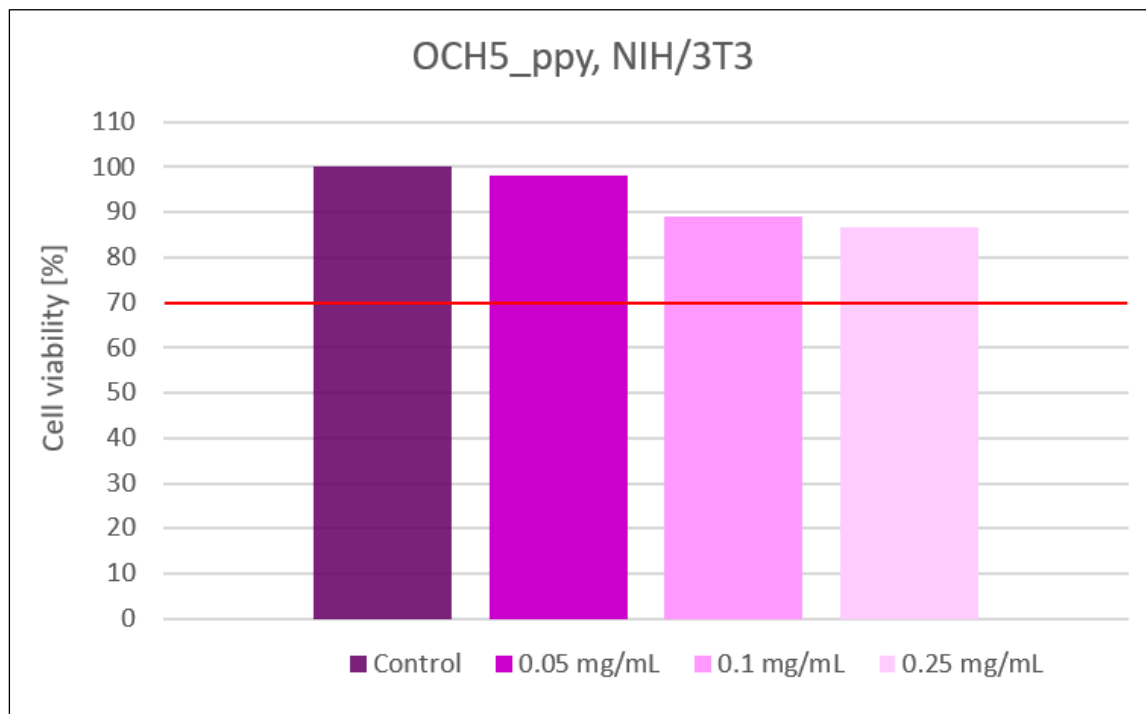


Figure 22 Cell viability of NIH/3T3 fibroblasts treated with OCH5_ppy sample, expressed relative to untreated control cells. The red line indicates the cytotoxicity threshold at 70% viability.

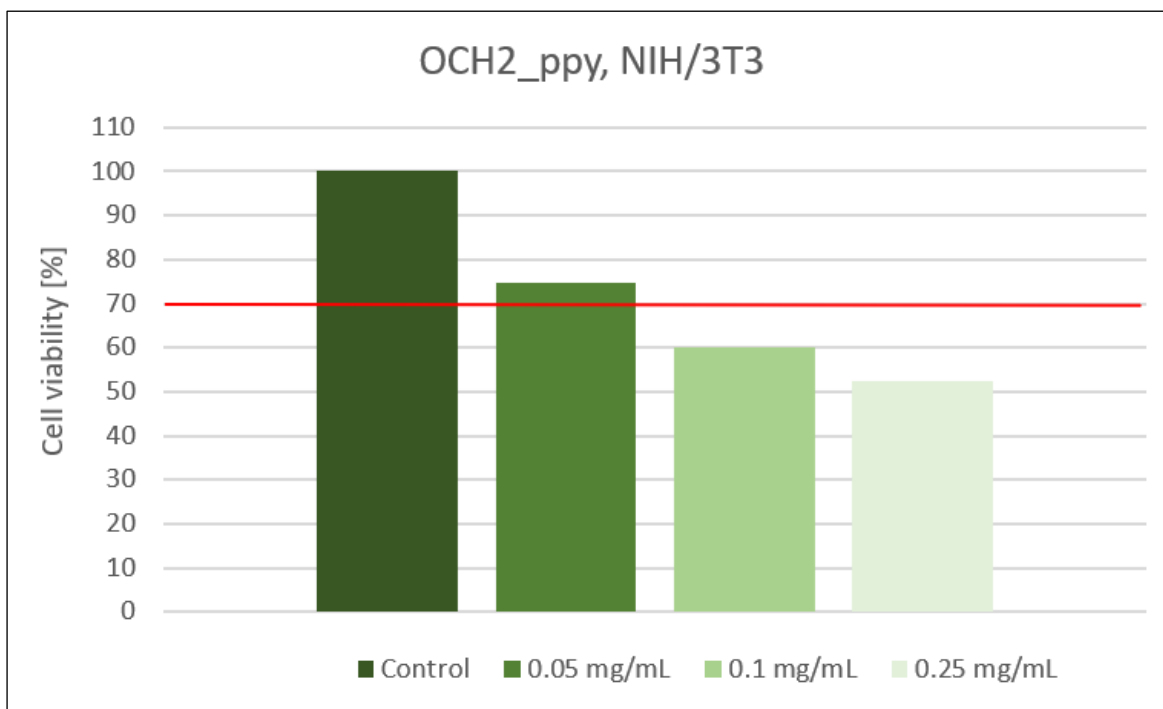


Figure 23 Cell viability of NIH/3T3 fibroblasts treated with OCH2_ppy sample, expressed relative to untreated control cells. The red line indicates the cytotoxicity threshold at 70% viability.

OCH9_ppy treated cells maintained high viability across all tested concentrations, with values consistently well above 80% (Figure 20). This sample also exhibited a positive zeta potential (15.4 mV) and a narrow particle size distribution (PdI = 0.5), suggesting a well-stabilized and uniform colloid. These features likely contributed to the excellent cytocompatibility observed, as stable, well-dispersed nanoparticles are less likely to induce cellular stress or membrane damage.

OCH8_ppy (octamer) displayed moderate cytocompatibility, with viability values decreasing slightly at higher concentrations but remaining above 70% (Figure 21). This sample featured low PdI (0.4) and a zeta potential of 13.93 mV, indicating good colloidal stability. The results suggest that OCH8_ppy is generally well tolerated by fibroblasts, particularly at lower concentrations, making it a promising candidate for applications requiring both conductivity and biocompatibility.

Cells treated with OCH5_ppy (pentamer) retained viabilities above the cytotoxicity threshold but showed a gradual decline at higher concentrations (Figure 22). This aligns with the low zeta potential (0.53 mV) and high polydispersity (PdI = 1.0) observed in DLS measurements, indicating a tendency toward aggregation. The shorter chain length of the pentamer may result in less effective surface stabilization, leading to particle clustering and

mild cytotoxicity at increased doses. However, the observed concentrations were well above the cytotoxicity threshold.

OCH2_ppy (dimer) induced the most pronounced cytotoxic response, with cell viability dropping below 70% at concentrations ≥ 0.1 mg/mL (Figure 23). This correlates with its negative zeta potential (-0.18 mV) and high polydispersity ($PdI = 1.0$), indicating poor colloidal stability and a high likelihood of aggregation. Its short chain length and low zeta potential appear to compromise biocompatibility. It is possible that poorly stabilized aggregates interact unfavorably with cell membranes or interfere with cellular metabolic activity, particularly at higher doses.

Overall, these results confirm a relationship between chitoooligosaccharide chain length, colloidal stability, and cytocompatibility. Longer chains (OCH9_ppy, OCH8_ppy, OCH5_ppy) lead to better surface stabilization, higher zeta potential, and greater cytocompatibility. Shorter chains (OCH2_ppy) result in less effective stability, greater polydispersity, and reduced cell viability, particularly at higher concentrations. These findings support the use of longer chitoooligosaccharide chains in the design of conductive, biocompatible colloidal systems for biomedical applications such as tissue engineering and wound healing.

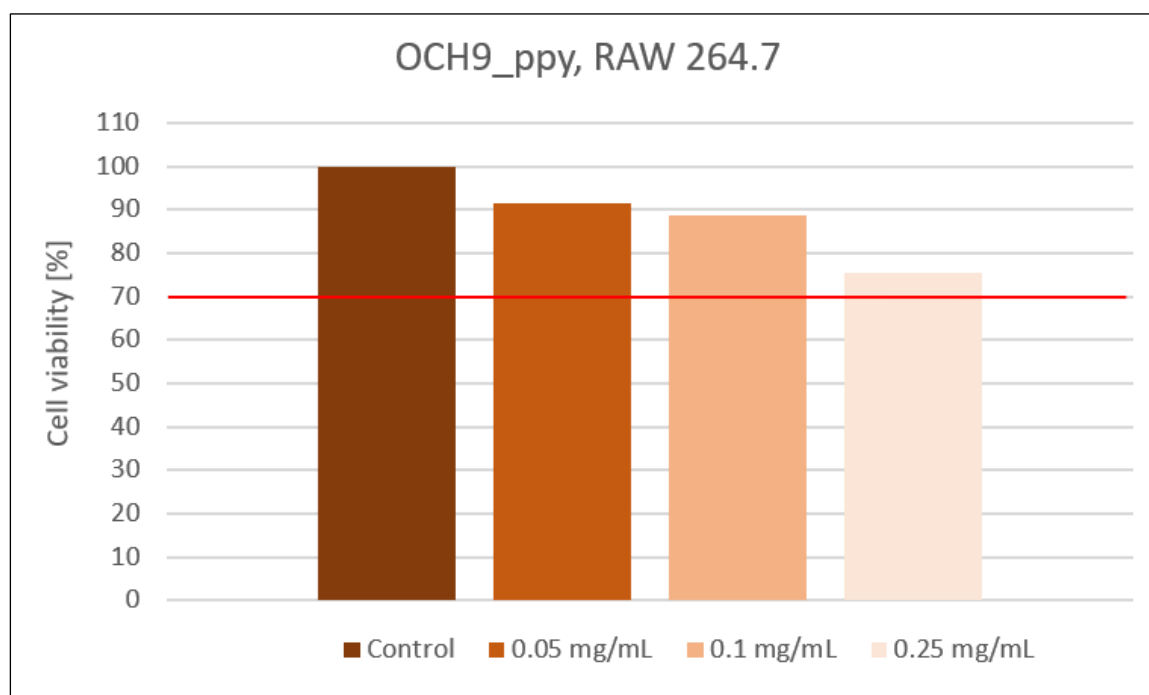


Figure 24 Cell viability of RAW 264.7 macrophages treated with OCH9_ppy sample and LPS, expressed relative to cells treated with LPS only. The red line indicates the cytotoxicity threshold at 70% viability.

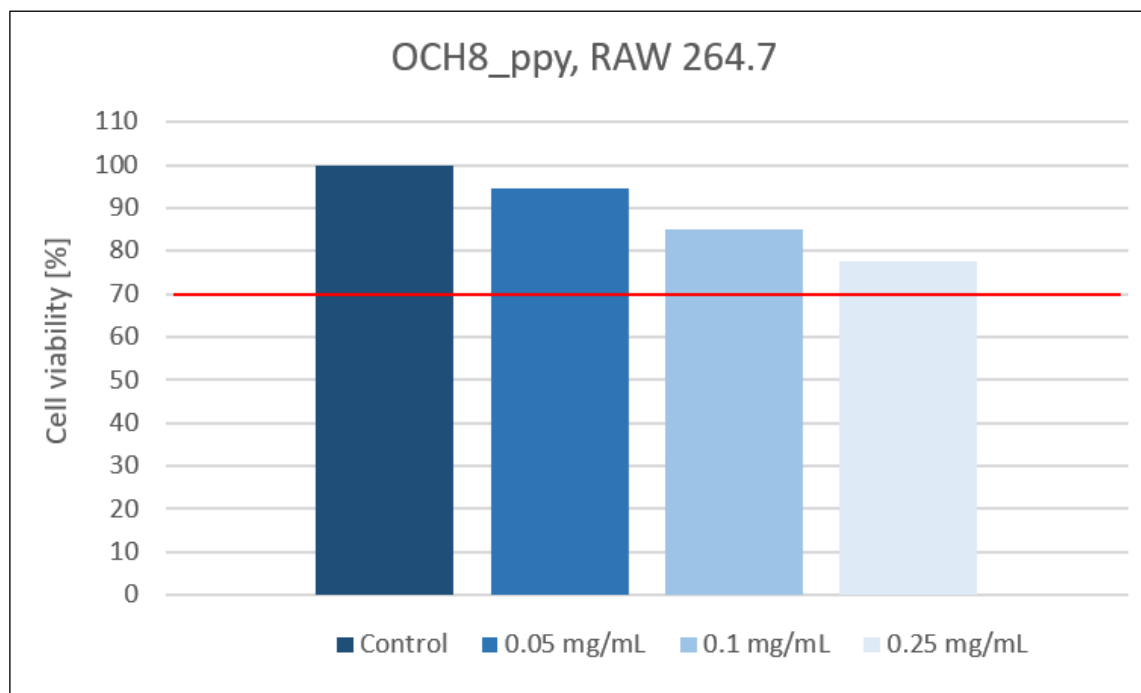


Figure 25 Cell viability of RAW 264.7 macrophages treated with OCH8_ppy sample and LPS, expressed relative to cells treated with LPS only. The red line indicates the cytotoxicity threshold at 70% viability.

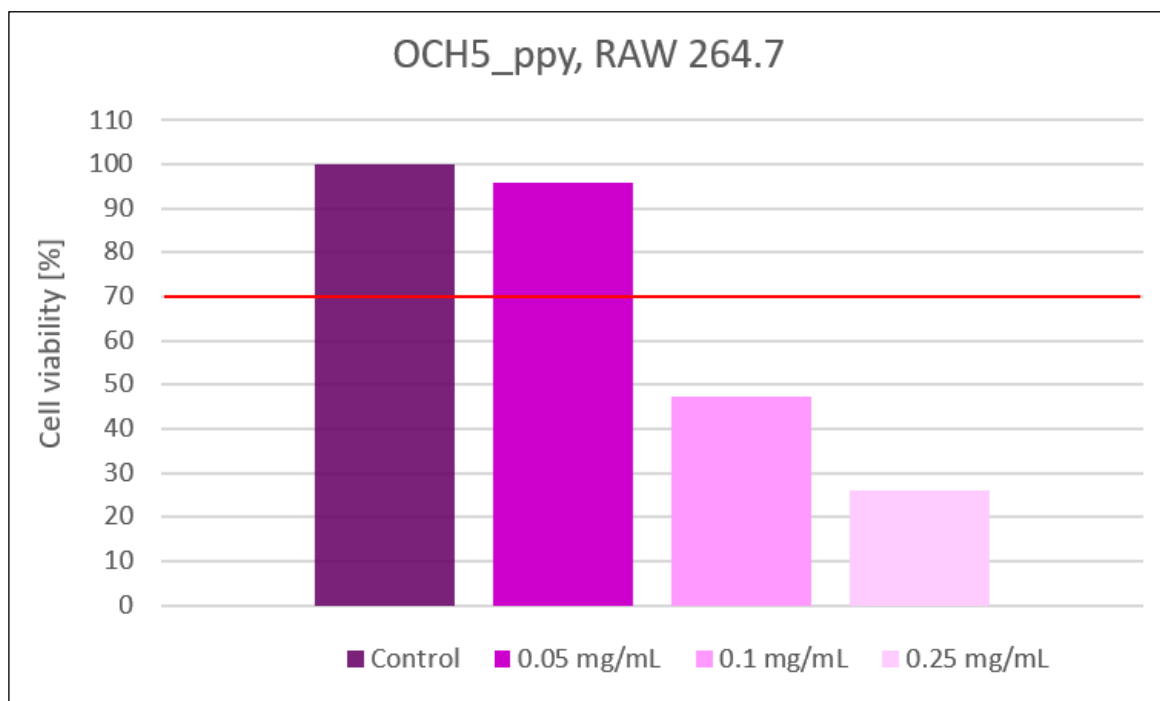


Figure 26 Cell viability of RAW 264.7 macrophages treated with OCH5_ppy sample and LPS, expressed relative to cells treated with LPS only. The red line indicates the cytotoxicity threshold at 70% viability.

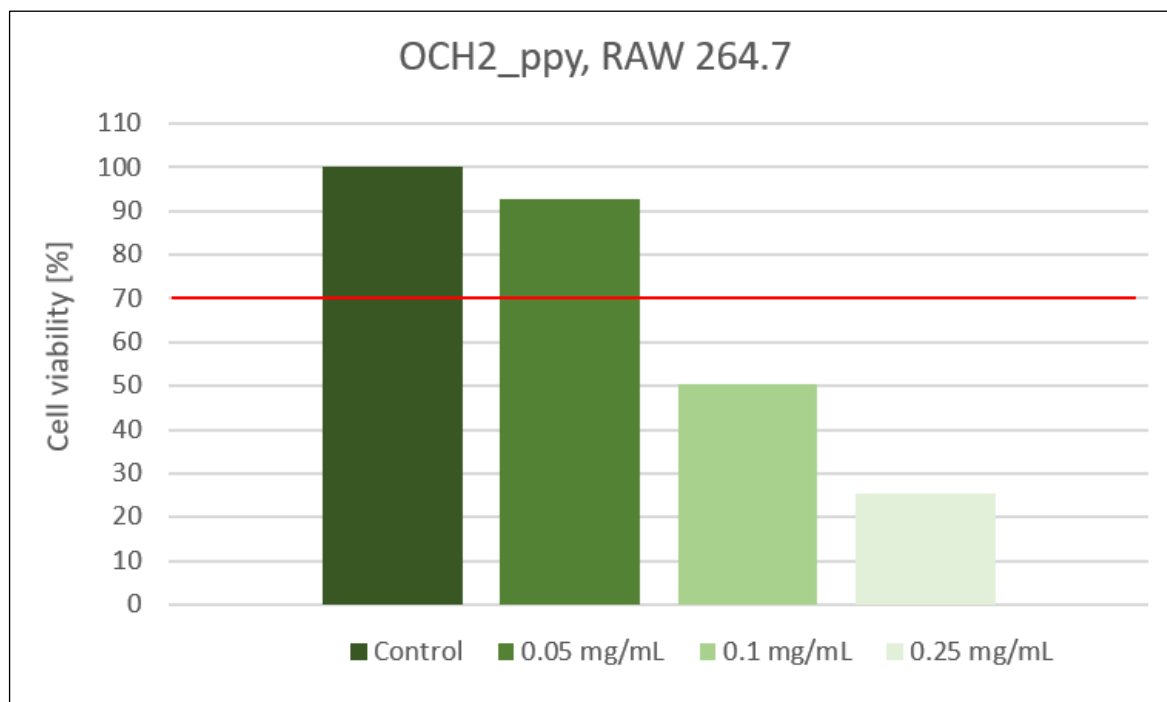


Figure 27 Cell viability of RAW 264.7 macrophages treated with OCH2_ppy sample and LPS, expressed relative to cells treated with LPS only. The red line indicates the cytotoxicity threshold at 70% viability.

The viability of RAW 264.7 macrophages following exposure to polypyrrole–chitoooligosaccharide colloidal particles was calculated relative to LPS-treated control cells to simulate an immune-stimulated baseline. A viability below 70%, marked by the red line in Figures 24–27, is considered cytotoxic. This assay also indirectly reflects how particle composition and physicochemical properties may influence immunocompetent cells.

As shown in Figure 24, OCH9_ppy treated macrophages maintained viability above 70% at concentrations up to 0.1 mg/mL. A slight reduction occurred at the highest concentration (0.25 mg/mL), but cell viability remained above the threshold. The relatively high zeta potential (15.4 mV) and low polydispersity ($PdI = 0.5$) indicate a stable colloid with minimal aggregation. These properties likely contributed to the moderate cytocompatibility observed, with minimal disruption to macrophage function at lower concentrations.

As seen in Figure 25, OCH8_ppy induced a concentration-dependent decrease in viability, though the decline was more gradual than that observed for OCH5_ppy. Viability remained above 70% up to 0.1 mg/mL, falling just below at the highest concentration. With a zeta potential of 13.93 mV and a low PdI of 0.4, OCH8_ppy formed relatively stable colloids. Its longer chitoooligosaccharide chains likely improved surface interactions,

resulting in better cytocompatibility compared to OCH5_ppy, though high concentrations may still pose a mild cytotoxic risk.

OCH5_ppy (Figure 26) exhibited marked cytotoxicity at higher concentrations, with cell viability dropping sharply below 50% at 0.1 mg/mL and under 30% at 0.25 mg/mL. Despite showing strong UV/VIS absorbance and good spectral characteristics, its low zeta potential (0.53 mV) and high PDI (1.0) indicate a highly heterogeneous particle population prone to aggregation. These aggregates may have led to enhanced cell stress or membrane interaction, triggering cytotoxic responses at elevated doses. The moderate chain length might have offered only limited stabilization, insufficient for macrophage compatibility in the higher concentration range.

Among all samples, OCH2_ppy exhibited the highest cytotoxicity, with viability falling below 70% already at 0.1 mg/mL and reaching nearly 25% at 0.25 mg/mL (Figure 27). Although this sample showed the highest polypyrrole content based on UV/VIS intensity and had a prominent 480 nm peak, its negative zeta potential (−0.18 mV) and high PDI (1.0) suggest poor colloidal stability and a strong tendency toward aggregation. The very short chain length appears insufficient for effective steric or electrostatic stabilization, making OCH2_ppy the least biocompatible sample in this context.

These results demonstrate a clear trend linking particle stability and composition to macrophage viability. Longer chitooligosaccharide chains (OCH9_ppy, OCH8_ppy) offer better stabilization, resulting in higher cell viability at lower concentrations. Shorter chains (OCH5_ppy, OCH2_ppy) yield less stable colloids prone to aggregation, contributing to cytotoxic effects, particularly at higher concentrations. The zeta potential and PDI values reflect the viability outcomes, reinforcing the importance of surface charge and particle uniformity in predicting biocompatibility.

The cytotoxic responses observed in macrophages, especially at higher doses, may also relate to immune sensitivity, which differs from fibroblasts. These findings highlight the critical role of biopolymer structure and colloidal characteristics in modulating cellular responses in immune-relevant cell lines.

To evaluate the immunomodulatory potential of the prepared polypyrrole–chitooligosaccharide colloidal particles, a nitrite production assay was conducted using the Griess reagent. This colorimetric assay measures accumulated nitrite (NO_2^-), a stable end-product of nitric oxide (NO), which is a key inflammatory mediator produced by activated

macrophages. The experiment was performed on RAW 264.7 cells, a murine macrophage cell line, which was co-stimulated with lipopolysaccharide (LPS) to mimic an inflammatory environment. (12, 70)

The aim of this assay was to determine whether the colloidal samples enhance or suppress NO production in comparison to LPS-stimulated controls, thereby indicating their pro- or anti-inflammatory activity. Nitrite concentration was therefore expressed relative to cells treated with LPS (Figures 28–31). Similarly to MTT assay, an additional control consisting of completely untreated macrophages was assessed to verify the baseline viability of the cell passages used, however, these results are not included in the data discussed in this thesis, as they served solely as a quality control checkpoint. Outliers excluded using Grubbs' test at a significance level of $\alpha = 0.05$.

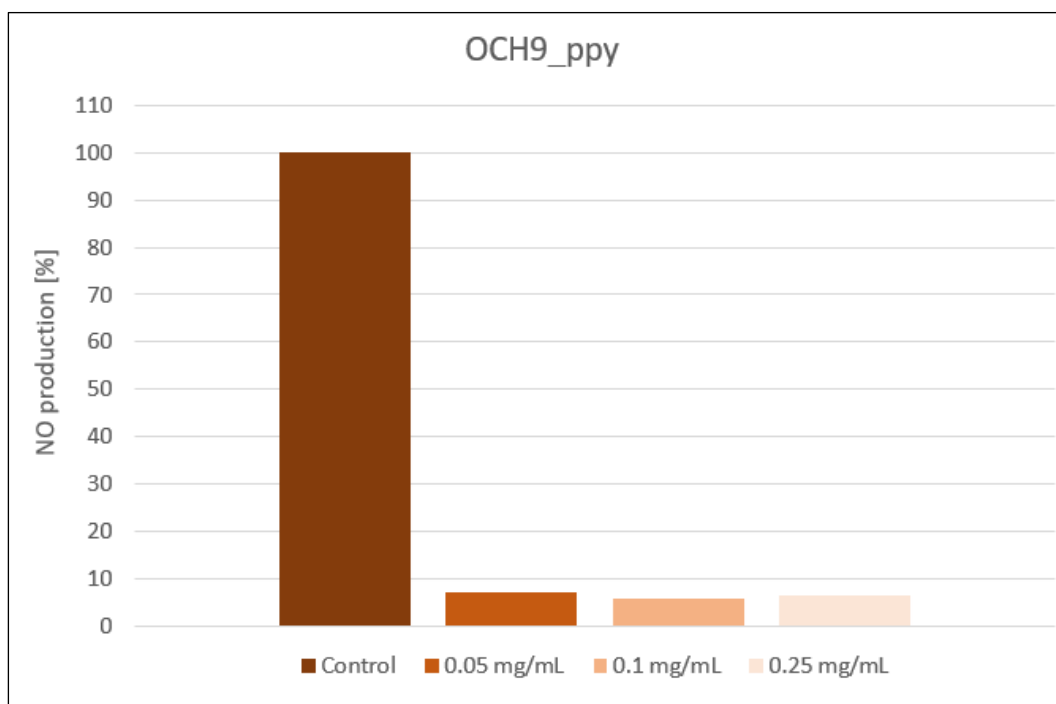


Figure 28 Nitric oxide production of RAW 264.7 macrophages treated with OCH9_ppy sample and LPS, expressed relative to cells treated with LPS only.

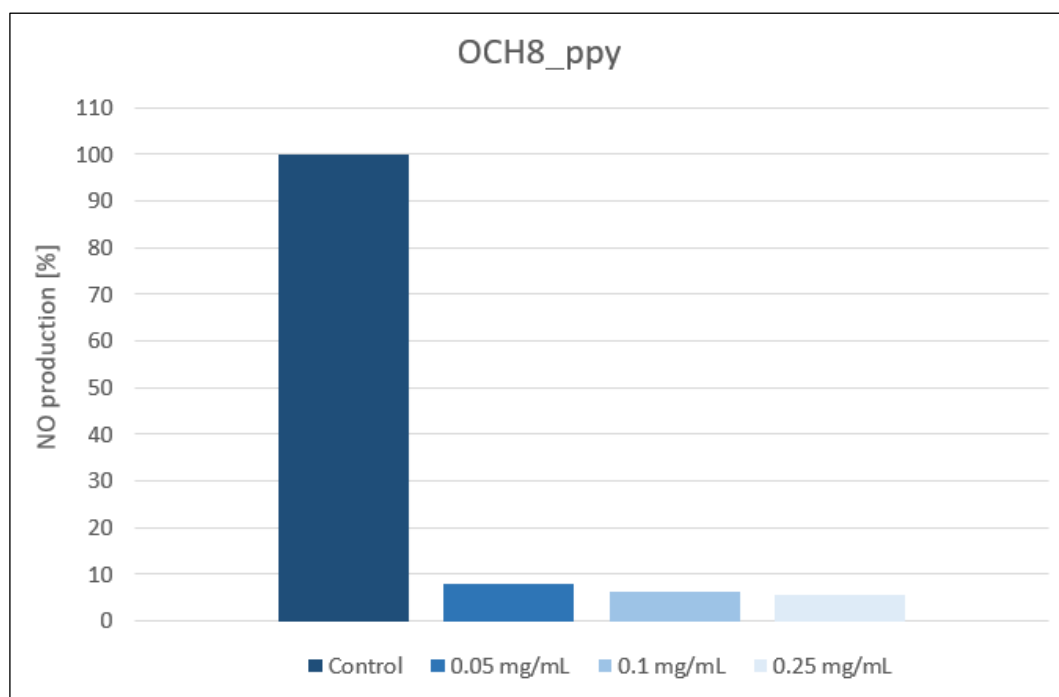


Figure 29 Nitric oxide production of RAW 264.7 macrophages treated with OCH8_ppy sample and LPS, expressed relative to cells treated with LPS only.

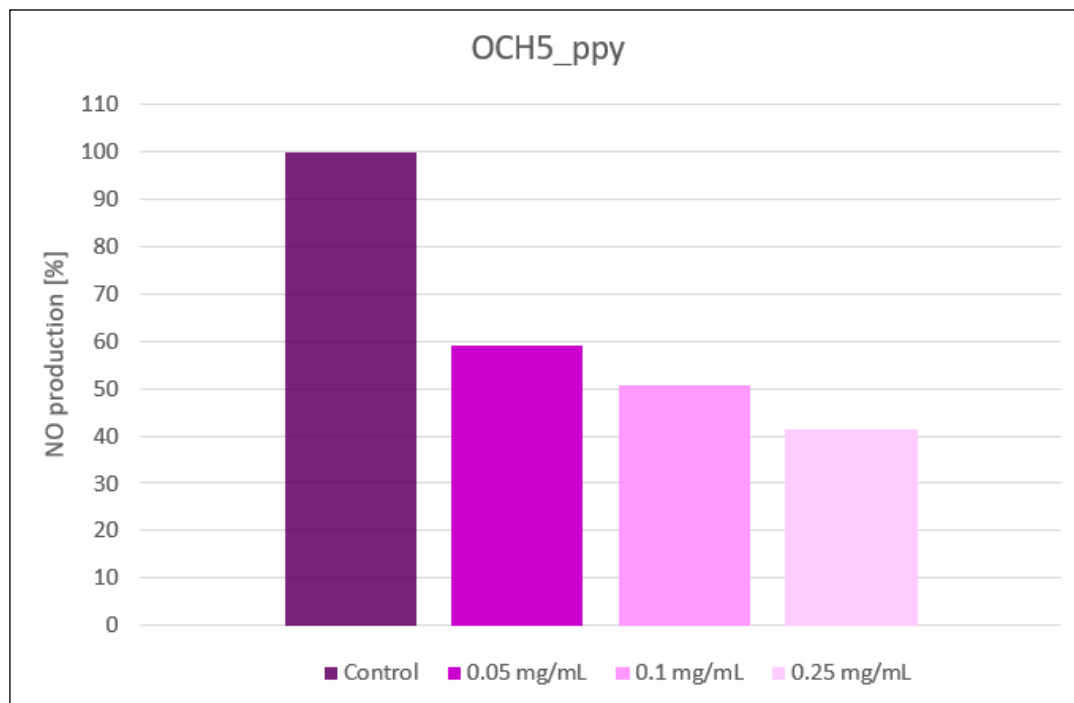


Figure 30 Nitric oxide production of RAW 264.7 macrophages treated with OCH5_ppy sample and LPS, expressed relative to cells treated with LPS only.

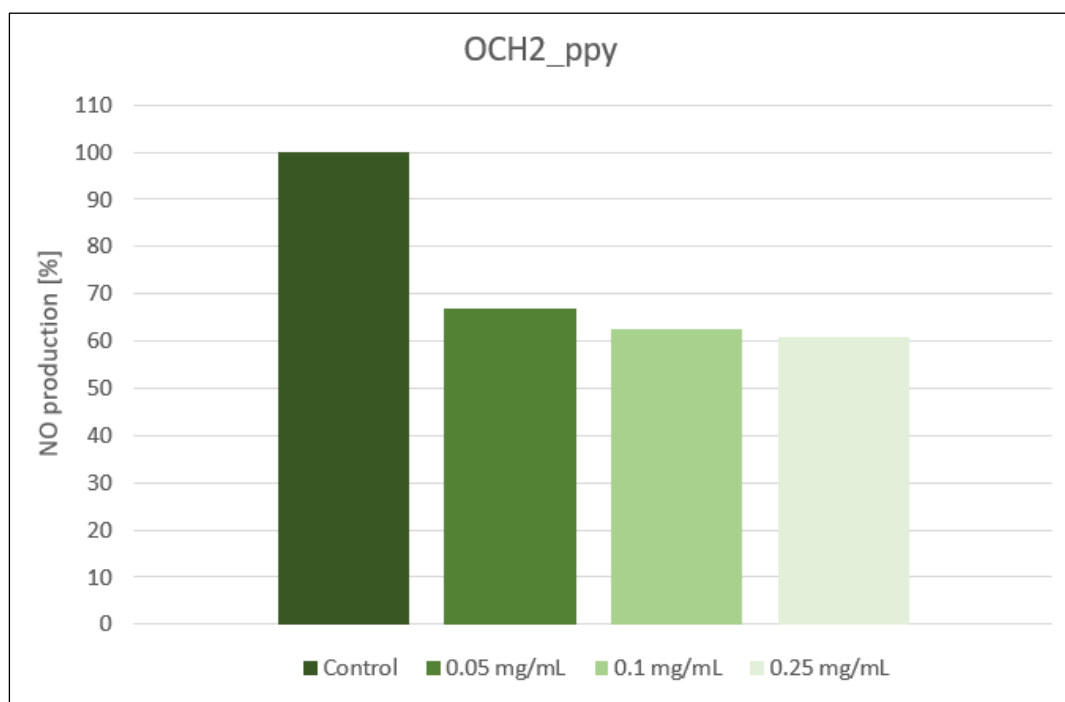


Figure 31 Nitric oxide production of RAW 264.7 macrophages treated with OCH2_ppy sample and LPS, expressed relative to cells treated with LPS only.

Figure 28 (OCH9_ppy) shows a drastic reduction in NO production across all tested concentrations. This suggests a strong anti-inflammatory effect, potentially due to the stable colloidal nature of OCH9_ppy and its excellent cytocompatibility observed in the MTT assay. The longer chitooligosaccharide chains likely formed stable, well-dispersed particles that do not activate or stress macrophages, even inhibiting LPS-induced NO production.

Figure 29 shows that OCH8_ppy, similarly to OCH9_ppy, induced a significant suppression of NO production even at the lowest tested concentration. These results support the interpretation that well-stabilized colloids with moderate biopolymer chain length can reduce inflammatory signaling without impairing viability as the MTT assay confirmed that cell viability remained above 70% up to 0.1 mg/mL.

As shown in Figure 30, OCH5_ppy led to a moderate but concentration-dependent reduction in NO levels, however the suppression is not as pronounced as with OCH9_ppy or OCH8_ppy. Notably, MTT data showed substantial cytotoxicity at concentrations ≥ 0.1 mg/mL, which likely contributes to reduced NO output at higher doses due to loss of viable cells.

For OCH2_ppy, as shown in Figure 31, NO production decreased only partially, with nitrite levels remaining around 60–70% even at the highest concentration. This is consistent with the cytotoxicity pattern observed in the MTT assay, where viability dropped sharply at

higher doses. OCH2_ppy had the lowest zeta potential and highest PDI, indicating extremely poor colloidal stability and likely particle aggregation. Thus, the observed moderate suppression of NO likely reflects the effect of cytotoxicity, rather than true anti-inflammatory activity.

These results align with earlier findings and confirm the pattern described above. Colloidal particles with longer chains, higher surface charge, and better dispersion, show strong anti-inflammatory activity via suppression of LPS-induced NO production. OCH5_py demonstrates an intermediate and dose-dependent suppression. OCH2_ppy with the shortest chitooligosaccharide chain, while somewhat suppressive, likely achieves this through cell damage rather than beneficial immunomodulation.

The data reinforce the importance of biopolymer chain length, particle stability, and cytocompatibility in predicting immunological responses to colloidal materials. These findings are linked to potential applications targeting inflammation-sensitive environments, such as tissue regeneration or immune-modulatory coatings.

To further investigate the immunomodulatory properties of the polypyrrole–chitooligosaccharide colloidal particles, an enzyme-linked immunosorbent assay (ELISA) was performed to quantify the secretion of interleukin-6 (IL-6) by RAW 264.7 macrophages. IL-6 is a key pro-inflammatory cytokine produced in response to immune stimuli such as lipopolysaccharide (LPS) and serves as a marker of macrophage activation and inflammatory signaling. (70, 71)

Cells were stimulated with LPS in the presence of colloidal samples at various concentrations. This assay complements the nitric oxide (NO) production data and provides additional insight into how the particles may influence cytokine-mediated immune responses. IL-6 concentration was expressed relative to cells treated with LPS only (Figures 32–35).

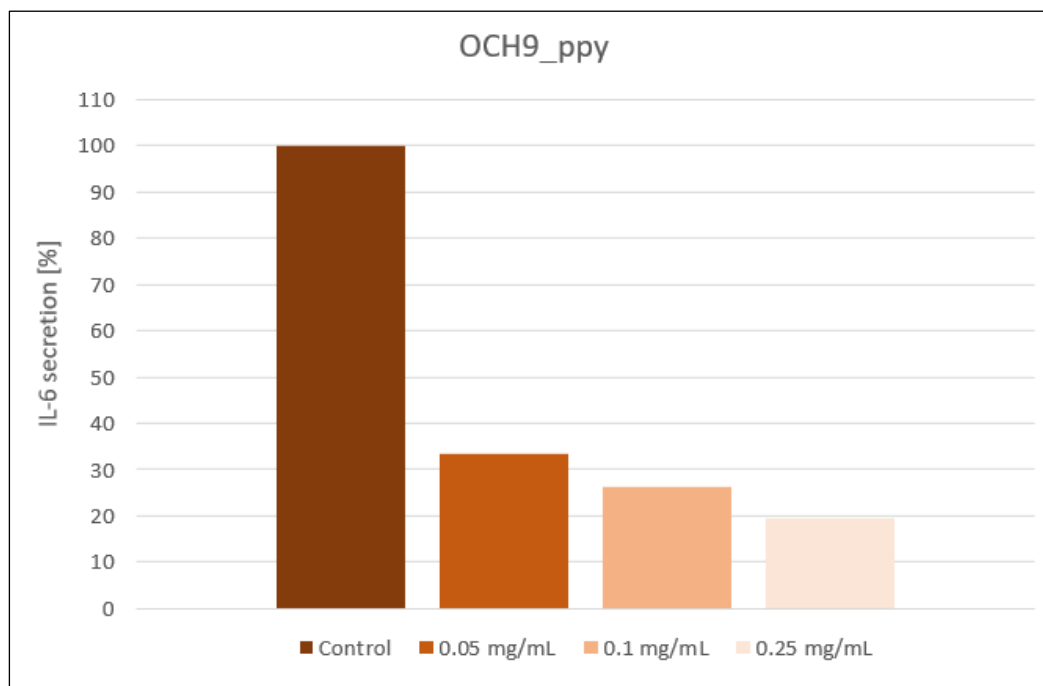


Figure 32 IL-6 secretion of RAW 264.7 macrophages treated with OCH9_ppy sample and LPS, expressed relative to cells treated with LPS only.

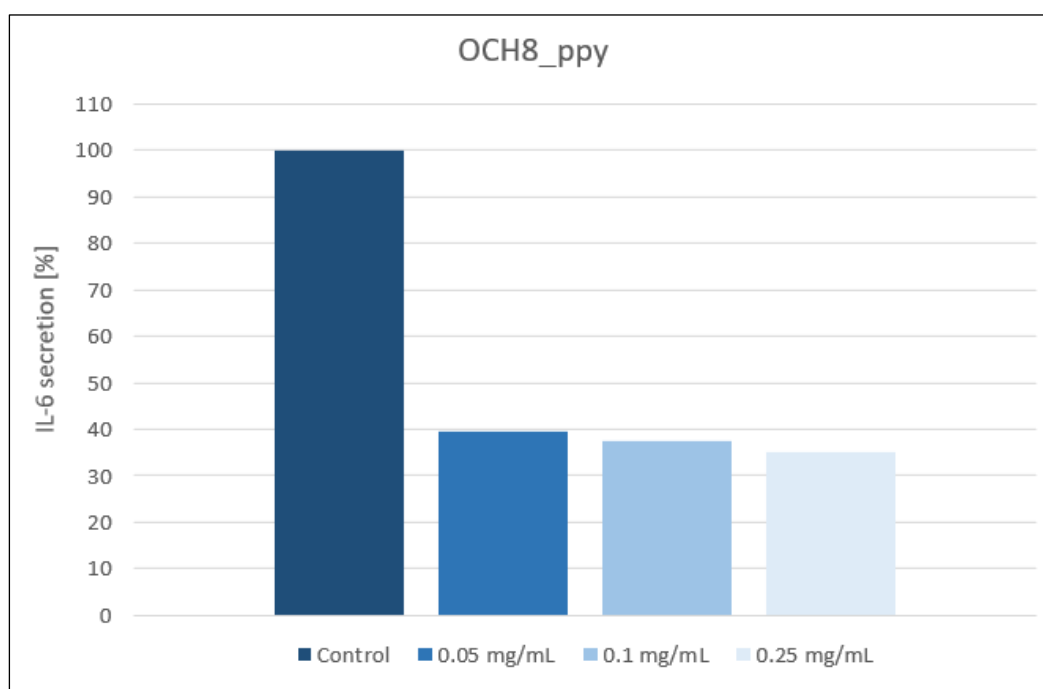


Figure 33 IL-6 secretion of RAW 264.7 macrophages treated with OCH8_ppy sample and LPS, expressed relative to cells treated with LPS only.

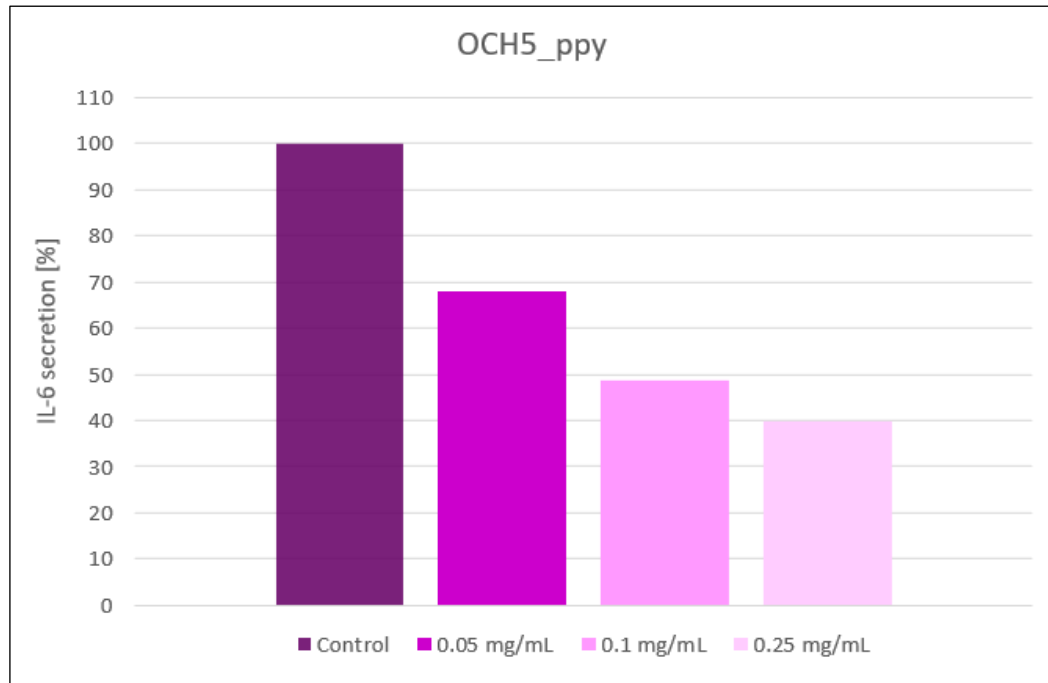


Figure 34 IL-6 secretion of RAW 264.7 macrophages treated with OCH5_ppy sample and LPS, expressed relative to cells treated with LPS only.

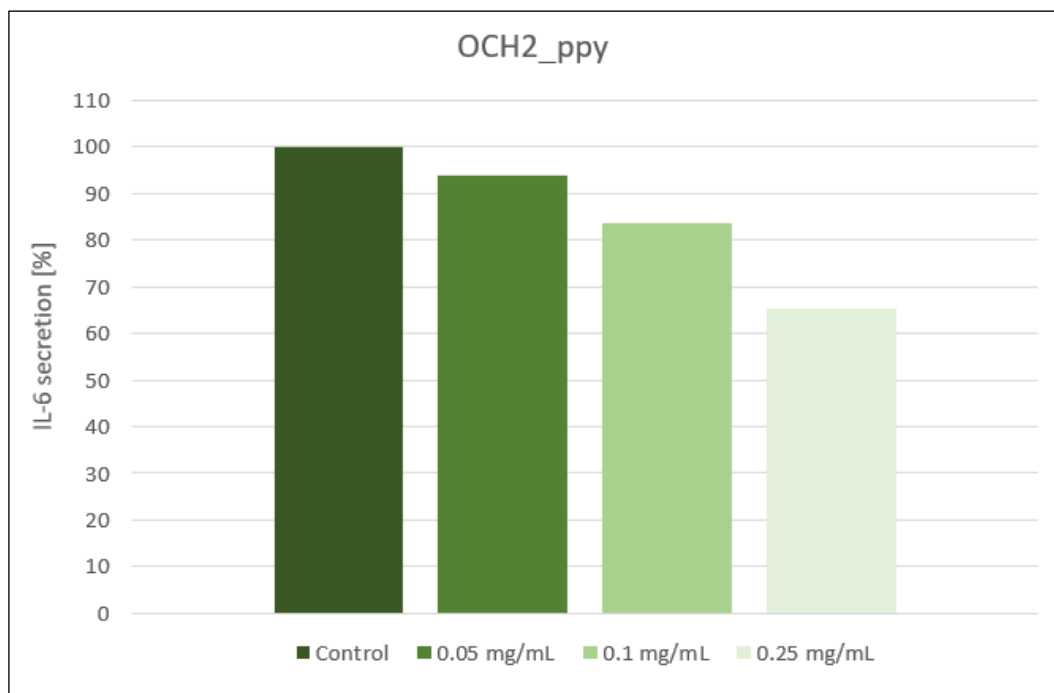


Figure 35 IL-6 secretion of RAW 264.7 macrophages treated with OCH2_ppy sample and LPS, expressed relative to cells treated with LPS only.

As shown in Figure 32, IL-6 levels with OCH9_ppy were strongly suppressed in a concentration-dependent manner, dropping to approximately 20% of the LPS control at the highest dose. This aligns with earlier results showing strong NO suppression. These findings

suggest that OCH9_ppy does not trigger inflammatory signaling and may even exhibit anti-inflammatory properties.

IL-6 levels for OCH8_ppy (Figure 33) also showed a consistent and strong reduction, comparable to OCH9. Given that the MTT assay indicated good cytocompatibility up to 0.1 mg/mL, this supports the conclusion that OCH8_ppy is non-immunogenic and likely anti-inflammatory.

Figure 34 (OCH5_ppy) demonstrates a gradual decrease in IL-6 secretion with increasing concentration. At lower doses, the reduction may reflect a mild immunomodulatory effect, but at higher doses (0.1 and 0.25 mg/mL), this is likely influenced by cytotoxicity, as shown in the MTT assay, where cell viability dropped well below 50%. The suppression of IL-6 here may therefore result from cell loss rather than true inhibition of immune signaling.

Unlike the other samples, OCH2_ppy (Figure 35) showed only a moderate reduction in IL-6 levels, remaining above 70% at lower concentrations. These findings align with the moderate NO suppression and clear cytotoxicity from the MTT assay. The weaker effect on IL-6 is likely due to the instability of this sample (low/negative zeta potential, high PdI), which results in partial immune activation at lower doses and cell damage-induced suppression at higher concentrations.

The IL-6 results reinforce the trends observed in both the MTT and NO assays. These data again underline the importance of biopolymer chain length, colloidal stability, and cytocompatibility in modulating inflammatory responses, supporting the use of longer oligosaccharides in the design of immunologically safe, biofunctional colloidal systems.

CONCLUSION

The goal of this thesis was to synthesize colloidal particles combining polypyrrole and chitooligosaccharides using an oxidant-free approach, and to assess their physicochemical characteristics, biocompatibility, and immunomodulatory potential. The theoretical part provided essential context for understanding the behavior of such hybrid materials. It first addressed the stability and characterization of colloidal systems, including stabilization mechanisms, the electrical double layer, and the use of microscopy and light scattering techniques. The section on conducting polymers focused on their synthesis (chemical and electrochemical), doping mechanisms, and their relevance in biomedical applications, particularly for wound healing and tissue engineering. Lastly, the biopolymer section examined biopolymer classification, biocompatibility, biodegradability, and the specific properties of chitosan and chitooligosaccharides, including their antioxidant, antimicrobial, and immunomodulatory effects.

The aim of the analysis was to prepare and characterize colloidal particles composed of polypyrrole and chitooligosaccharides of varying chain lengths, and to evaluate their physicochemical properties, cytocompatibility, and immunomodulatory effects *in vitro*.

Dynamic Light Scattering (DLS) analysis revealed that the colloidal stability and size distribution were strongly influenced by chitooligosaccharide chain length. Particles prepared with longer chains (OCH9_ppy – nonamer, and OCH8_ppy – octamer) displayed intermediate size distributions ($PDI \leq 0.5$), moderate average diameters, and positive zeta potentials (approximately 13–15 mV), indicative of well-dispersed and electrostatically stable systems. In contrast, samples with shorter chains (OCH5_ppy – pentamer, and especially OCH2_ppy – dimer) were characterized by high polydispersity, lower or even negative zeta potentials, and likely a strong tendency to form aggregates.

The UV/VIS spectra confirmed the successful incorporation of polypyrrole across all samples, with characteristic absorbance peaks near 290 nm and additional features around 480 nm depending on polymer content. Notably, OCH2_ppy showed the most intense absorption, correlating with its higher polypyrrole content.

Cytocompatibility, assessed via MTT assay, demonstrated that OCH9_ppy and OCH8_ppy maintained high viability in both NIH/3T3 fibroblasts and RAW 264.7 macrophages across all tested concentrations, indicating low cytotoxicity and good cellular tolerance. In contrast, OCH5_ppy showed dose-dependent cytotoxicity, and OCH2_ppy

significantly reduced cell viability, particularly in macrophages, with values falling below the 70% cytotoxicity threshold even at lower concentrations. These results suggest that colloidal stability and biopolymer chain length are critical for biocompatibility.

Immunomodulatory activity was investigated through NO production (Griess assay) and IL-6 secretion (ELISA) in RAW 264.7 macrophages. Samples OCH9_ppy and OCH8_ppy caused strong suppression of both NO and IL-6 levels, indicating anti-inflammatory or biologically inert behavior, likely due to their stable, well-dispersed structures and minimal interaction with inflammatory pathways. OCH5_ppy led to partial suppression of these markers, especially at higher doses, although this was likely attributable to cytotoxicity rather than true immunoregulation. OCH2_ppy showed minimal suppression of NO and IL-6 at lower concentrations and more pronounced reductions at higher concentrations, likely corresponding with a loss of viable immune cells due to colloidal instability and particle aggregation, similar to OCH5_ppy.

In conclusion, this study demonstrates that the chain length of chitooligosaccharides used to stabilize polypyrrole colloidal particles plays a critical role in determining physicochemical stability, cellular compatibility, and immune response. The nonamer- and octamer-based systems (OCH9_ppy and OCH8_ppy) emerged as the most promising candidates for further biomedical application, offering a favorable balance between conductive functionality, biocompatibility, and immunological safety. These findings highlight the potential of oxidant-free, green synthesis strategies for creating biofunctional colloids tailored for use in tissue engineering, drug delivery, or wound healing platforms.

BIBLIOGRAPHY

1. KINGSLEY OGEMDI, Iwuozor. Properties and Uses of Colloids: A Review. *Colloid and Surface Science*. 2019. Vol. 4, no. 2, p. 24. DOI 10.11648/j.css.20190402.12.
2. GOŁĘBIEWSKI, Adrian and BUSZEWSKI, Bogusław. Characterization of colloidal particles of a biological and metallic nature. *Microchemical Journal*. 2023. Vol. 191, p. 108864. DOI 10.1016/j.microc.2023.108864.
3. SPIELMAN-SUN, Eleanor, BOYE, Kristin, DWIVEDI, Dipankar, ENGEL, Maya, THOMPSON, Aaron, KUMAR, Naresh and NOËL, Vincent. A Critical Look at Colloid Generation, Stability, and Transport in Redox-Dynamic Environments: Challenges and Perspectives. *ACS Earth and Space Chemistry*. 18 April 2024. Vol. 8, no. 4, p. 630–653. DOI 10.1021/acsearthspacechem.3c00255.
4. BALINT, Richard, CASSIDY, Nigel J. and CARTMELL, Sarah H. Conductive polymers: Towards a smart biomaterial for tissue engineering. *Acta Biomaterialia*. June 2014. Vol. 10, no. 6, p. 2341–2353. DOI 10.1016/j.actbio.2014.02.015.
5. FUJII, Syuji. Synthesis of Conductive Polymer-based Composite Particles. *Chemistry Letters*. 2023. Vol. 52, no. 8, p. 631–639. DOI 10.1246/cl.230235.
6. K, Namsheer and ROUT, Chandra Sekhar. Conducting polymers: a comprehensive review on recent advances in synthesis, properties and applications. *RSC Advances*. 2021. Vol. 11, no. 10, p. 5659–5697. DOI 10.1039/D0RA07800J.
7. UPADHYE, Mohini, KUCHEKAR Mohini, PUJARI, Rohini Revansiddhappa, and SABLE, Nutan Uttam. Biopolymers: A comprehensive review. *Research Journal of Science and Technology*. 2022. Vol. 4, no. 1, p. 013–018. DOI 10.53022/oarjst.2022.4.1.0070.
8. HASSAN, Mohamed, BAI, Jun and DOU, De-Qiang. Biopolymers; Definition, Classification and Applications. *Egyptian Journal of Chemistry*. 2019. DOI 10.21608/ejchem.2019.6967.1580.
9. HARUGADE, Anuja, SHERJE, Atul P. and PETHE, Anil. Chitosan: A review on properties, biological activities and recent progress in biomedical applications. *Reactive and Functional Polymers*. 2023. Vol. 191, p. 105634. DOI 10.1016/j.reactfunctpolym.2023.105634.
10. LIAQAT, Fakhra and ELTEM, Rengin. Chitooligosaccharides and their biological activities: A comprehensive review. *Carbohydrate Polymers*. 2018. Vol. 184, p. 243–259. DOI 10.1016/j.carbpol.2017.12.067.
11. HIMANSHU, SAMEEKSHA and KUMAR, Mukesh. A Comprehensive Review on Pharmaceutical Liquid Dosage Form. *Acta Scientific Pharmaceutical Sciences*. 2022. p. 12–24. DOI 10.31080/ASPS.2022.06.0867.
12. KÁČEROVÁ, Simona, VÍCHOVÁ, Zdenka, VALÁŠKOVÁ, Kristýna, VÍCHA, Jan, MÜNSTER, Lukáš, KAŠPÁRKOVÁ, Věra, VAŠÍČEK, Ondřej and HUMPOLÍČEK, Petr. Biocompatibility of colloidal polypyrrole. *Colloids and Surfaces B: Biointerfaces*. 2023. Vol. 232, p. 113605. DOI 10.1016/j.colsurfb.2023.113605.

13. BAEK, Woonhyuk, CHANG, Hogeun, BOOTHARAJU, Megalamane S., KIM, Jeong Hyun, PARK, Sungjun and HYEON, Taeghwan. Recent Advances and Prospects in Colloidal Nanomaterials. *Journal of the American Chemical Society*. 2021. Vol. 1, no. 11, p. 1849–1859. DOI 10.1021/jacsau.1c00339.
14. CÖLFEN, H. and VÖLKEL, A., Analytical ultracentrifugation in colloid chemistry. *Analytical Ultracentrifugation VII*. Springer Berlin Heidelberg, 2004. p. 31–47. ISBN 978-3-540-20203-5.
15. CHEN, Jiann-Jy, LUI, Chun-Chung, CHEN, Tien-Yu, TSENG, Ping-Tao and HUNG, Chao-Ming. The Tyndall Effect in High-Resolution Computed Tomography of Semicircular Canalolithiasis with Benign Paroxysmal Positional Vertigo. *Diagnostics*. 2022. Vol. 12, no. 4, p. 1000. DOI 10.3390/diagnostics12041000.
16. MATUSIAK, Jakub and GRZĄDKA, Elżbieta. Stability of colloidal systems - a review of the stability measurements methods. *Annales Universitatis Mariae Curie-Skłodowska, section AA – Chemia*. 2017. Vol. 72, no. 1, p. 33. DOI 10.17951/aa.2017.72.1.33.
17. KINGSLEY OGEMDI, Iwuozor. Properties and Uses of Colloids: A Review. *Colloid and Surface Science*. 2019. Vol. 4, no. 2, p. 24. DOI 10.11648/j.css.20190402.12.
18. GHOSH, Aritra, BANDYOPADHYAY, Malay, DATTA GUPTA, Sushanta and GUPTA, Shamik. Independent-oscillator model and the quantum Langevin equation for an oscillator: A review. *J. Stat. Mech.* 2023. DOI 10.48550/ARXIV.2306.02665.
19. AGMO HERNÁNDEZ, Víctor. An overview of surface forces and the DLVO theory. *ChemTexts*. 2023. Vol. 9, no. 4. DOI 10.1007/s40828-023-00182-9.
20. BUSCHOW, K. H. J. (ed.). *Encyclopedia of materials: science and technology*. Amsterdam, New York : Elsevier, 2001. ISBN 978-0-08-043152-9.
21. NIKOLAEV, Boris, YAKOVLEVA, Ludmila, FEDOROV, Viacheslav, LI, Hanmei, GAO, Huile and SHEVTSOV, Maxim. Nano- and Microemulsions in Biomedicine: From Theory to Practice. *Pharmaceutics*. 2023. Vol. 15, no. 7, p. 1989. DOI 10.3390/pharmaceutics15071989.
22. LYKLEMA, J., VAN LEEUWEN, H.P. and MINOR, M. DLVO-theory, a dynamic re-interpretation. *Advances in Colloid and Interface Science*. 1999. Vol. 83, no. 1–3, p. 33–69. DOI 10.1016/s0001-8686(99)00011-1.
23. LIEBCHEN, Benno and MUKHOPADHYAY, Aritra K. Interactions in active colloids. *Journal of Physics: Condensed Matter*. 2022. Vol. 34, no. 8, p. 083002. DOI 10.1088/1361-648x/ac3a86.
24. CHHABRA, Raj, BASAVARAJ, Madivala G. Colloidal Dispersions. *Coulson and Richardson's Chemical Engineering. Volume 2A. Particulate systems and particle technology*. Elsevier, 2019. p. 693–737. ISBN 978-0-08-101098-3.
25. DOURADO, André H. B. Electric Double Layer: The Good, the Bad, and the Beauty. *Electrochem*. 2022. Vol. 3, no. 4, p. 789–808. DOI 10.3390/electrochem3040052.

26. WU, Jianzhong. Understanding the Electric Double-Layer Structure, Capacitance, and Charging Dynamics. *Chemical Reviews*. 2022. Vol. 122, no. 12, p. 10821–10859. DOI 10.1021/acs.chemrev.2c00097.
27. RODRIGUEZ-LOYA, Jesus, LERMA, Maricarmen and GARDEA-TORRESDEY, Jorge L. Dynamic Light Scattering and Its Application to Control Nanoparticle Aggregation in Colloidal Systems: A Review. *Micromachines*. 2023. Vol. 15, no. 1, p. 24. DOI 10.3390/mi15010024.
28. HASSAS, Behzad Vaziri. Effect of hydrophilic lipophilic balance (HLB) of nonionic surfactants on ultrafine lignite dewatering. *Thesis for B.Sc. Columbia University*. 2013. DOI 10.12140/RG.2.1.3600.8721
29. KONTOGEORGIS, Georgios M. and KIIL, Søren. *Introduction to Applied Colloid and Surface Chemistry*. Wiley, 2016. p. 202-210. ISBN 978-1-118-88118-7.
30. GALLEGOS-CERDA, Susana Dianey, HERNÁNDEZ-VARELA, Josué David, ARREDONDO-TAMAYO, Benjamín and CHANONA PÉREZ, José Jorge. A review of advanced microscopy techniques for the development of nanotechnology in agriculture, food, and the environment. *Mundo Nano. Revista Interdisciplinaria en Nanociencias y Nanotecnología*. 2022. Vol. 16, no. 30, p. 1–33. DOI 10.22201/ceiich.24485691e.2023.30.69723.
31. MÜLLER-GOYMANN, C.C. Physicochemical characterization of colloidal drug delivery systems such as reverse micelles, vesicles, liquid crystals and nanoparticles for topical administration. *European Journal of Pharmaceutics and Biopharmaceutics*. 2004. Vol. 58, no. 2, p. 343–356. DOI 10.1016/j.ejpb.2004.03.028.
32. MEKURIA, Tewodros Abere and KINDE, Mebrie Zemene. Basic Principles and Applications of Live Cell Microscopy Techniques: A Review. *World's Veterinary Journal*. 2022. p. 339–346. DOI 10.54203/scil.2022.wvj43.
33. GUEYE, Magatte N., CARELLA, Alexandre, FAURE-VINCENT, Jérôme, DEMADRILLE, Renaud and SIMONATO, Jean-Pierre. Progress in understanding structure and transport properties of PEDOT-based materials: A critical review. *Progress in Materials Science*. 2020. Vol. 108, p. 100616. DOI 10.1016/j.pmatsci.2019.100616.
34. RAHIMZADEH, Zahra, NAGHIB, Seyed Morteza, ZARE, Yasser and RHEE, Kyong Yop. An overview on the synthesis and recent applications of conducting poly(3,4-ethylenedioxythiophene) (PEDOT) in industry and biomedicine. *Journal of Materials Science*. 2020. Vol. 55, no. 18, p. 7575–7611. DOI 10.1007/s10853-020-04561-2.
35. PANG, Ai Ling, ARSAD, Agus and AHMADIPOUR, Mohsen. Synthesis and factor affecting on the conductivity of polypyrrole: a short review. *Polymers for Advanced Technologies*. 2021. Vol. 32, no. 4, p. 1428–1454. DOI 10.1002/pat.5201. i
36. TALIKOWSKA, Milena, FU, Xiaoxu and LISAK, Grzegorz. Application of conducting polymers to wound care and skin tissue engineering: A review. *Biosensors and Bioelectronics*. 2019. Vol. 135, p. 50–63. DOI 10.1016/j.bios.2019.04.001.
37. MAO, Jifu and ZHANG, Ze. Polypyrrole as Electrically Conductive Biomaterials: Synthesis, Biofunctionalization, Potential Applications and Challenges. In : CHUN, Heung

Jae, PARK, Chan Hum, KWON, Il Keun and KHANG, Gilson (eds.), *Cutting-Edge Enabling Technologies for Regenerative Medicine*. Singapore : Springer Singapore, 2018. p. 347–370. Advances in Experimental Medicine and Biology. ISBN 978-981-13-0949-6.

38. BOCCHETTA, Patrizia, FRATTINI, Domenico, TAGLIENTE, Miriana and SELLERI, Filippo. Electrochemical Deposition of Polypyrrole Nanostructures for Energy Applications: A Review. *Current Nanoscience*. 2020. Vol. 16, no. 4, p. 462–477. DOI 10.2174/1573413715666190717113600.

39. BREDAS, Jean Luc and STREET, G. Bryan. Polarons, bipolarons, and solitons in conducting polymers. *Accounts of Chemical Research*. 1985. Vol. 18, no. 10, p. 309–315. DOI 10.1021/ar00118a005.

40. IYER, Nandini V., AGAWANE, Ganesh L., PATEL, Chandan, KHER, Jayant A. and BHAME, Shekhar D. Contemporary Advances in Metal Oxide Doped Polypyrrole Composites: A Comprehensive Review. *International Journal of Nanoscience and Nanotechnology*. 2024. Vol. 20, no. 2. DOI 10.22034/ijnn.2024.2005501.2391.

41. JADOUN, Sapana, RATHORE, Dharendra Singh, RIAZ, Ufana and CHAUHAN, Narendra Pal Singh. Tailoring of conducting polymers via copolymerization – A review. *European Polymer Journal*. 2021. Vol. 155, p. 110561. DOI 10.1016/j.eurpolymj.2021.110561.

42. ATEH, D.D, NAVSARIA, H.A and VADGAMA, P. Polypyrrole-based conducting polymers and interactions with biological tissues. *Journal of The Royal Society Interface*. 2006. Vol. 3, no. 11, p. 741–752. DOI 10.1098/rsif.2006.0141.

43. XIE, Qi and WANG, Changhong. Polyacetylenes in herbal medicine: A comprehensive review of its occurrence, pharmacology, toxicology, and pharmacokinetics. *Phytochemistry*. 2022. Vol. 201, p. 113288. DOI 10.1016/j.phytochem.2022.113288.

44. SHIRAKAWA, Hideki. Synthesis and characterization of highly conducting polyacetylene. *Synthetic Metals*. 1995. Vol. 69, no. 1–3, p. 3–8. DOI 10.1016/0379-6779(94)02340-5.

45. GUO, Baolin and MA, Peter X. Conducting Polymers for Tissue Engineering. *Biomacromolecules*. 2018. Vol. 19, no. 6, p. 1764–1782. DOI 10.1021/acs.biomac.8b00276.

46. SONG, Ziyu, WANG, Jiajun, TAN, Shaojie, GAO, Jing and WANG, Lu. Conductive biomimetic bilayer fibrous scaffold for skin regeneration. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2023. Vol. 656, p. 130211. DOI 10.1016/j.colsurfa.2022.130211.

47. AMIN, Usman, KHAN, Muhammad Usman, MAJEED, Yaqoob, REBEZOV, Maksim, KHAYRULLIN, Mars, BOBKOVA, Elena, SHARIATI, Mohammad Ali, CHUNG, Ill Min and THIRUVENGADAM, Muthu. Potentials of polysaccharides, lipids and proteins in biodegradable food packaging applications. *International Journal of Biological Macromolecules*. 2021. Vol. 183, p. 2184–2198. DOI 10.1016/j.ijbiomac.2021.05.182.

48. BISWAS, Manik Chandra, JONY, Bodiuzzaman, NANDY, Pranab Kumar, CHOWDHURY, Reaz Ahmed, HALDER, Sudipta, KUMAR, Deepak, RAMAKRISHNA,

Seeram, HASSAN, Masud, AHSAN, Md Ariful, HOQUE, Md Enamul and IMAM, Muhammad Ali. Recent Advancement of Biopolymers and Their Potential Biomedical Applications. *Journal of Polymers and the Environment*. 2022. Vol. 30, no. 1, p. 51–74. DOI 10.1007/s10924-021-02199-y.

49. PEERS, S., MONTEMBault, A. and LADAVIÈRE, C. Chitosan hydrogels incorporating colloids for sustained drug delivery. *Carbohydrate Polymers*. 2022. Vol. 275, p. 118689. DOI 10.1016/j.carbpol.2021.118689.

50. JUIKAR, Subodh K. and WARKAR, Sudhir G. Biopolymers for packaging applications: An overview. *Packaging Technology and Science*. April 2023. Vol. 36, no. 4, p. 229–251. DOI 10.1002/pts.2707.

51. SHIROUD HEIDARI, Behzad, RUAN, Rui, VAHABLI, Ebrahim, CHEN, Peilin, DE-JUAN-PARDO, Elena M., ZHENG, Minghao and DOYLE, Barry. Natural, synthetic and commercially-available biopolymers used to regenerate tendons and ligaments. *Bioactive Materials*. 2023. Vol. 19, p. 179–197. DOI 10.1016/j.bioactmat.2022.04.003.

52. JABEEN, Nazish and ATIF, Muhammad. Polysaccharides based biopolymers for biomedical applications: A review. *Polymers for Advanced Technologies*. 2024. Vol. 35, no. 1. DOI 10.1002/pat.6203.

53. RANGANADHAREDDY, Aluru and CHANDRSEKHAR, Chanda. Polyhydroxyalkanoates, the Biopolymers of Microbial Origin - A Review. *Journal of Biochemical Technology*. 2022. Vol. 13, no. 3, p. 1–6. DOI 10.51847/3qf2wvuzl2.

54. SHARMA, Mohit, TELLILI, Nihed, KACEM, Imen and ROUISSI, Tarek. Microbial Biopolymers: From Production to Environmental Applications - A Review. *Applied Sciences*. 2024. Vol. 14, no. 12, p. 5081. DOI 10.3390/app14125081.

55. JOSHI, Jnanada Shrikant, LANGWALD, Sarah Vanessa, EHRMANN, Andrea and SABANTINA, Lilia. Algae-Based Biopolymers for Batteries and Biofuel Applications in Comparison with Bacterial Biopolymers - A Review. *Polymers*. 2024. Vol. 16, no. 5, p. 610. DOI 10.3390/polym16050610.

56. BARANWAL, Anupriya, KUMAR, Ashutosh, PRIYADHARSHINI, A., OGGU, Gopi Suresh, BHATNAGAR, Ira, SRIVASTAVA, Ananya and CHANDRA, Pranjali. Chitosan: An undisputed bio-fabrication material for tissue engineering and bio-sensing applications. *International Journal of Biological Macromolecules*. 2018. Vol. 110, p. 110–123. DOI 10.1016/j.ijbiomac.2018.01.006.

57. XIE, Yitong, GAO, Shishuai, ZHANG, Daihui, WANG, Chunpeng and CHU, Fuxiang. Bio-based polymeric materials synthesized from renewable resources: A mini-review. *Resources Chemicals and Materials*. 2023. Vol. 2, no. 3, p. 223–230. DOI 10.1016/j.recmm.2023.05.001.

58. LODHI, Gaurav, KIM, Yon-Suk, HWANG, Jin-Woo, KIM, Se-Kwon, JEON, You-Jin, JE, Jae-Young, AHN, Chang-Bum, MOON, Sang-Ho, JEON, Byong-Tae and PARK, Pyo-Jam. Chitoooligosaccharide and Its Derivatives: Preparation and Biological Applications. *BioMed Research International*. 2014. Vol. 2014, p. 1–13. DOI 10.1155/2014/654913.

59. LIANG, Shuang, SUN, Yaxuan and DAI, Xueling. A Review of the Preparation, Analysis and Biological Functions of Chitooligosaccharide. *International Journal of Molecular Sciences*. 2018. Vol. 19, no. 8, p. 2197. DOI 10.3390/ijms19082197.
60. ALMINDER EJ, Fahad M. and EL-GHOUL, Yassine. Synthesis and study of a new biopolymer-based chitosan/hematoxylin grafted to cotton wound dressings. *Journal of Applied Polymer Science*. 2019. Vol. 136, no. 23. DOI 10.1002/app.47625.
61. G. SANDRI, S. Rossi, BONFERONI, M.C., FERRARI, F., MORI, M. and CAMELLA, C. The role of chitosan as a mucoadhesive agent in mucosal drug delivery. *Journal of Drug Delivery Science and Technology*. 2012. Vol. 22, no. 4, p. 275–284. DOI 10.1016/S1773-2247(12)50046-8.
62. KE, Cai-Ling, DENG, Fu-Sheng, CHUANG, Chih-Yu and LIN, Ching-Hsuan. Antimicrobial Actions and Applications of Chitosan. *Polymers*. 2021. Vol. 13, no. 6, p. 904. DOI 10.3390/polym13060904.
63. YILMAZ ATAY, Hüsnügül. Antibacterial Activity of Chitosan-Based Systems. In : JANA, Sougata and JANA, Subrata (eds.), *Functional Chitosan*. Singapore : Springer Singapore, 2019. p. 457–489. ISBN 978-981-15-0262-0.
64. IVANOVA, Donika G. and YANEVA, Zvezdelina L. Antioxidant Properties and Redox-Modulating Activity of Chitosan and Its Derivatives: Biomaterials with Application in Cancer Therapy. *BioResearch* 2020. Vol. 9, no. 1, p. 64–72. DOI 10.1089/biores.2019.0028.
65. ANRAKU, Makoto, GEBICKI, Janusz M., IOHARA, Daisuke, TOMIDA, Hisao, UEKAMA, Kaneto, MARUYAMA, Toru, HIRAYAMA, Fumitoshi and OTAGIRI, Masaki. Antioxidant activities of chitosans and its derivatives in in vitro and in vivo studies. *Carbohydrate Polymers*. 2018. Vol. 199, p. 141–149. DOI 10.1016/j.carbpol.2018.07.016.
66. NIU, Wei, DONG, Yuelin, FU, Ziwei, LV, Jiajie, WANG, Ligui, ZHANG, Zhenhai, HUO, Jiege and JU, Jianming. Effects of molecular weight of chitosan on anti-inflammatory activity and modulation of intestinal microflora in an ulcerative colitis model. *International Journal of Biological Macromolecules*. 2021. Vol. 193, p. 1927–1936. DOI 10.1016/j.ijbiomac.2021.11.024.
67. AZUMA, Kazuo, OSAKI, Tomohiro, MINAMI, Saburo and OKAMOTO, Yoshiharu. Anticancer and Anti-Inflammatory Properties of Chitin and Chitosan Oligosaccharides. *Journal of Functional Biomaterials*. 2015. Vol. 6, no. 1, p. 33–49. DOI 10.3390/jfb6010033.
68. YUAN, Xiaojiao and REMITA, Hynd. Conjugated Polymer Polypyrrole Nanostructures: Synthesis and Photocatalytic Applications. *Topics in Current Chemistry*. 2022. Vol. 380, no. 5, p. 32. DOI 10.1007/s41061-022-00388-4.
69. DEY, Smita and KUMAR KAR, Asit. Morphological and Optical Properties of Polypyrrole Nanoparticles Synthesized by Variation of Monomer to Oxidant Ratio. *Materials Today: Proceedings*. 2019. Vol. 18, p. 1072–1076. DOI 10.1016/j.matpr.2019.06.566.

70. VASICEK, Ondrej, RUBANOVA, Daniela, CHYTKOVA, Barbora and KUBALA, Lukas. Natural pseurotins inhibit proliferation and inflammatory responses through the inactivation of STAT signaling pathways in macrophages. *Food and Chemical Toxicology*. 2020. Vol. 141, p. 111348. DOI 10.1016/j.fct.2020.111348.
71. VERESCAKOVA, Hana, AMBROZOVA, Gabriela, KUBALA, Lukas, PERECKO, Tomas, KOUDELKA, Adolf, VASICEK, Ondrej, RUDOLPH, Tanja K., KLINKE, Anna, WOODCOCK, Steven R., FREEMAN, Bruce A. and PEKAROVA, Michaela. Nitro-oleic acid regulates growth factor-induced differentiation of bone marrow-derived macrophages. *Free Radical Biology and Medicine*. 2017. Vol. 104, p. 10–19. DOI 10.1016/j.freeradbiomed.2017.01.003.

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
°C	degree Celsius
Cl ⁻	chlorine anion
cm	centrimetre
CO ₂	carbon dioxide
DA	degree of acetylation
Da	Dalton
DD	degree of deacetylation
DLS	dynamic light scattering
DLVO	Derjaguin–Landau–Verwey–Overbeek theory
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
E-SEM	environmental scanning electron microscopy
FBS	fetal bovine serum
g/mol	grams per mole
HCl	hydrochloric acid
HDPE	high-density polyethylene
¹ H NMR	proton nuclear magnetic resonance spectroscopy
hrs	hours
H ₂ SO ₄	sulphuric acid
IL-6	interleukin-6
L	litre
LDPE	low-density polyethylene

LPS	lipopolysaccharide
mg	milligram
mg/mL	milligram per millilitre
mRNA	messenger ribonucleic acid
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
mS/cm	milliSiemens per centimetre
mV	millivolt
MW	molecular weight
MWCO	molecular weight cut-off
N ₂	nitrogen gas
NaCl	sodium chloride
NaNO ₂	sodium nitrite
NIH/3T3	murine fibroblast cell line
NO	nitric oxide
nm	nanometre
OCH1	ch
OCH3	ch
OCH5	ch
OCH6	ch
OCH1_ppy	o
OCH3_ppy	o
OCH5_ppy	o
OCH6_ppy	o
PA	polyamide
PANI	polyaniline
PBS	phosphate buffered saline

PdI	polydispersity index
PEDOT	poly(3,4-ethylenedioxythiophene)
PES	poly(ether sulfone)
PF	polyfuran
PP	polypropylene
PPy	polypyrrol
PPV	poly(phenylene vinylene)
PSS	poly(styrene sulfonate)
PTH	polythiophene
PVC	poly(vinyl chloride)
RAW 264.7	murine macrophage cell line
RNA	ribonucleic acid
SAXS	small angle x-ray scattering
SEM	scanning electron microscopy
TEM	transmission electron microscopy
UPW	ultrapure water
UV/VIS	ultraviolet/visible
µm	micrometre, micron

