# Enzyme-catalyzed polymerization process: A novel approach to the preparation of polyaniline colloidal dispersions with an immunomodulatory effect

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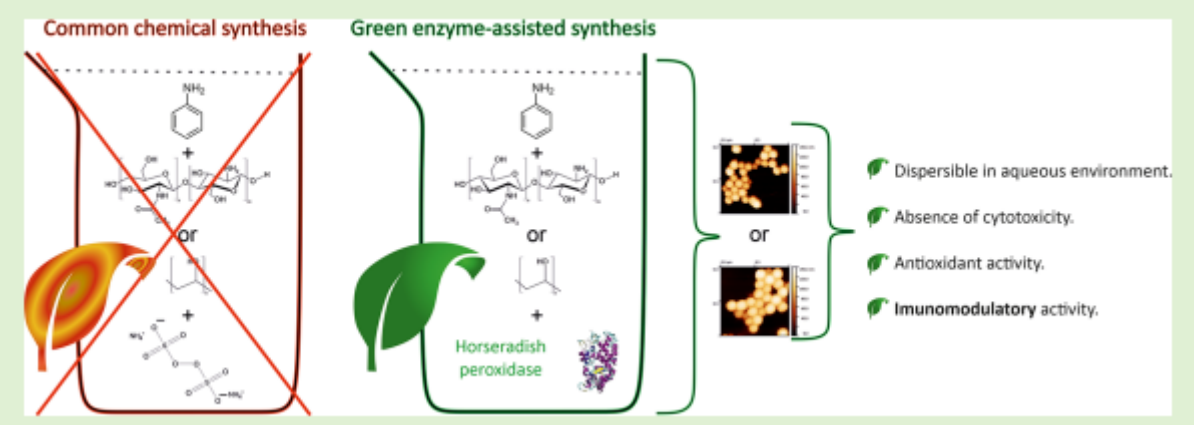


**ABSTRACT**: A green, nature-friendly synthesis of polyaniline colloidal particles based on enzyme-assisted oxidation of aniline with horseradish peroxidase and chitosan or poly(vinyl alcohol) as steric stabilizers was successfully employed. Physicochemical characterization revealed formation of particles containing the polyaniline emeraldine salt and demonstrated only a minor effect of polymer stabilizers on particle morphology. All tested colloidal particles showed in vitro antioxidation activity determined via scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. In vitro, they were able to reduce oxidative stress and inhibit the production of reactive oxygen species by neutrophils and inflammatory cytokines by macrophages. The anti-inflammatory effect observed was related to their antioxidant activity, especially in the case of neutrophils. The particles can thus be especially advantageous as active components of biomaterials modulating the early stages of inflammation. In addition to the immunomodulatory effect, the presence of intrinsically conducting polyaniline can impart cell-instructive properties to the particles. The approach to particle synthesis that we employed—an original one using environmentally friendly and biocompatible horseradish peroxidase—represents a smart way of preparing conducting particles with unique properties, which can be further modified by the stabilizers used.

## INTRODUCTION

The discovery of intrinsically conducting polymers (ICPs) opened the door to research of an attractive new material exhibiting interesting properties and suggesting numerous possibilities with respect to its application. Ever-increasing numbers of papers dealing with ICPs document interest in these materials. Polyaniline (PANI) is an ICP that has been investigated with regard to many applications, including biomedicine.[1-3] This polymer exhibits advantages, such as the ease and low cost of synthesis, the ability to electrically switch between its conductive and resistive states, and stability.

PANI is commonly prepared by the reaction of ammonium persulfate with aniline in an acidic aqueous medium, where PANI precipitates as a powder.[4] Unfortunately, such PANIpowder is insoluble and difficult to process. Although the solubility of PANI in an aqueous environment[5] or organic solvents[6] can be improved, the utilization of toxic organic solvents or severe functionalization conditions make these processes inconvenient for commercial purposes, increase the environmental burden, and raise

concerns about the biocompatibility of the product. One way to avoid these complications is to prepare water-dispersible PANI colloidal particles.

PANI colloids have received a lot of attention because of the potential use they could have in sensors, electrorheological fluids, and coatings. PANI colloids can be routinely synthesized by the polymerization of aniline in the presence of oxidizing agents and steric stabilizers, these including a wide variety of water-soluble polymers and biopolymers, such as poly(vinyl alcohol) (PVA)[7], poly(vinyl pyrrolidone)[8], and poly(2-acrylamido-2-methyl-1-propanesulfonic acid),[9] among others. In addition, some of them have shown low cytotoxicity, on the one hand,[10] and considerable antibacterial activity on the other.[11] In connection with biomedical applications, we can increasingly encounter the use of several biomacromolecules as stabilizers, such as cellulose derivatives, proteins, gelatine,[12] and chitosan,[13,14] the latter known for its unique antibacterial properties.

Application of enzymes, peroxidases, in the synthesis of colloidal PANI appears to be an efficient and environmentally friendly procedure, which can be an alternative to the chemical or electrochemical synthesis route of these systems. Recently, peroxidase-catalyzed oxidative polymerization was used by Jin et al.[15] for the preparation of a stable microemulsion or by Cruz-Silva et al.[16,17] , who prepared PANI colloidal particles with chitosan and PVA. The advantages of this enzyme-catalyzed method are the milder conditions under reaction where the oxidation rate mainly depends on the amount and activity of the enzyme; on the other hand, the common chemical oxidation of aniline is a non-autocatalytic reaction.[18] In addition, during enzymatic oxidation supported by hydrogen peroxide, only a limited concentration of potentially harmful byproducts (e.g., the contamination of the reaction media with salts) is formed,[19,20] and a limited increase in acidity is observed in comparison with the chemical route of synthesis.[21]

In this work, we prepared and characterized colloidal particles of PANI by an environmentally friendly approach based on the enzyme-assisted polymerization of aniline in aqueous media with the aid of horseradish peroxidase, using either chitosan or PVA as a steric stabilizer. The main novelty and originality of the work, however, lies in the examination of the biological properties of the prepared particles. On the basis of our previous knowledge[10] and published studies,[22] we anticipated that the colloidal particles would exhibit antioxidant properties, which could induce immunomodulatory effects on macrophages and neutrophils. The biological characterization also included cytotoxicity tests conducted on two cell lines, fibroblasts (NIH/3T3) and macrophages (RAW264.7).

## EXPERIMENTAL SECTION

*Materials.* Reagent-grade aniline (>98%) was purchased from PENTA (Czech Republic). Partially hydrolyzed (>99%) poly(vinyl alcohol) (PVA, Mw 89 000-98 000 g $moL^{-1}$), p-toluenesulfonic acid (TSA; >98.5%), high-molecular-weight chitosan (CHIT; molecular weight of 310 000-375 000 g $moL^{-1}$), and horseradish peroxidase (HRP; type VI, activity >250 U $g^{-1}$, RZ 3.2) were acquired from Sigma-Aldrich. Hydrogen peroxide (30%) was purchased from Kittford (Czech Republic).

*Preparation of Colloidal Particles*. Colloidal PANI was synthesized using both PVA and CHIT as steric stabilizers. The dispersion polymerization was carried out according to Cruz-Silva et al.[14] with minor modifications. Briefly, 1.2 g of PVA, 0.215 mL of aniline, and 0.456 g of TSA were dissolved in 20 mL of water. The reaction mixture was kept under vigorous stirring in a water/ice bath for 6 h. Afterward, 2.0 mL of a freshly prepared enzyme solution in Milli-Qwater (1.2 mg $mL^{-1}$) was added to the reaction mixture under stirring. The reaction was initiated by adding 2 mL of hydrogen peroxide (3.75 wt %). The hydrogen peroxide solution was added dropwise to the stirred reaction mixture over a time period

of2 h. The resulting polyaniline composite particles (PANI-PVA) were transferred into membrane tubing (MWCO 12 000-14 000; Spectrum Laboratories Inc.) and exhaustively dialyzed against 0.2 mol $L^{-1}$ hydrochloric acid to remove any residual monomers and byproducts. For the production of colloidal particles stabilized with chitosan (PANI-CHIT), 0.040 g of CHIT and 0.215 mL of aniline were added to 20 mL of Milli-Qwater, after which TSA was added slowly until a pH of 3.0 was achieved. The procedure for particle production was the same as that described above for PANI-PVA. A reference PANI sample (designated PANI-N) was prepared in a similar manner to PANI-PVA, however, without the PVA stabilizer.

*Size and Colloidal Stability of Particles.* The sizes and size distributions of the colloidal particles were determined on both freshly prepared and dialyzed samples by dynamic light scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern Instruments, U.K.). Measurements of the hydrodynamic radii of colloidal particles, expressed as z-average particle diameters, were performed at 25 °C. The intensity of scattered light (λ= 633 nm) was observed at a scattering angle of 173°. The polydispersity index (PDI) was calculated by assuming a lognormal distribution of particle sizes. Samples for analysis were prepared by diluting 10 $\mu$L of the colloidal dispersion in 1 mL of 0.1 mol $L^{-1}$ hydrochloric acid. All analyses were run in triplicate and are expressed as the mean and standard deviation (SD). Using the same procedure, the colloidal stability of the particles was determined after 24 months of storage at 5 ± 2 °C, and the z-average particle diameters and PDI were recorded and calculated.

*Antioxidation Activity Determined by Scavenging of DPPH Radicals.* Antioxidant activity of PANI-CHI and PANI-PVA was determined by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay[22] using a previously published method with modifications. Freshly prepared 1 X $10^{-4}$ M DPPH solution in ethanol (1 mL) was added to 10 $\mu$L of each of the investigated samples. The reaction mixture was stirred for 30 s and UV spectra were recorded within a time period of 60 min. The absorbance was read at 516 nm using a Jasco V-750 spectrophotometer (Jasco Inc.), and antioxidation activity was calculated as % DPPH scavenging activity = $[(A_0 — A_1/A_0]$ X 100, where $A_0$ and $A_1$ are absorbances of DPPH solution in the absence and the presence of tested samples, respectively. DPPH ethanolic solution served as a blank.

*UV—Vis Spectroscopy*. UV—vis spectra of PANI dispersions in a wavelength range of 200—800 nm were obtained on a UV—vis Varian Cary 300 instrument, Varian Inc. For sample preparation, a 10 $\mu$L sample was withdrawn and dispersed in 3.0 mL of 1.0 mol $L^{-1}$ hydrochloric acid or in Milli-Qwater. The UV—vis spectra of PANI dispersions recorded in 1.0 mol $L^{-1}$ hydrochloric acid were used for determining the concentration of PANI in the colloidal dispersions. This data was needed for testing the biological properties of samples. The concentration of PANI in each of the samples was calculated from the local maximum of absorbance at a wavelength of 395 nm using the Lambert—Beer law, A = $\varepsilon cl$, where e is the absorption coefficient ($\varepsilon$ = 31 500 ± 1700 $cm^2\ g^{-1}$),[23] $c$ is the concentration of PANI, and $l$ is the optical path ($l$ = 1 cm).

*Transmission Electron Microscopy.* The morphology of colloidal particles was assessed by means of a JEM 2100 transmission electron microscope (JEOL, Japan) using an accelerating voltage of 160 kV. Samples were prepared by the gentle drying of a diluted (0.1 wt %) sample dispersion drop-cast on a formvar-coated copper grid.

*Atomic Force Microscopy*. Stock particle dispersions were diluted 20 times with Milli-Qwater. In the following step, 20 $\mu$L of the diluted dispersion was deposited on a freshly cleaved mica surface. V-5 grade Mica (SPI) with an area of 10 X 10 $mm^2$ was used. After 5 min of exposure, mica-surface-attached colloids were gently dried using a stream of air. Dried colloids were observed with an AFM microscope, using the ScanAsyst mode on a Dimension ICON instrument (Bruker Corporation). Analyses were

performed under laboratory temperature and humidity. A ScanAsyst-Air silicon nitride probe (Bruker Corporation) with a spring constant of 0.4 N $m^{-1}$ was used. The scanning rate was 0.5 Hz. Data from the AFM were processed using Gwyddion 2.5 software (Czech Metrology Institute, Czech Republic).

*Cell Cultures and Cultivation Techniques*. Cytotoxicity testing was performed using the NIH/3T3 mouse embryonic fibroblast cell line (ECACC 93061524, England) and RAW264.7 murine peritoneal macrophage cell line (European Collection of Authenticated Cell Cultures, U.K.). Dulbecco's modified Eagle's medium (Biosera, France) with 10% calf serum (Biosera, France) and 100 mg mL-penicillin/streptomycin (Biosera, France) was used for the cultivation of NIH/3T3 cells. Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% heat-inactivated low endotoxin fetal bovine serum (FBS; PAN, Germany) and 1% combination of penicillin/streptomycin was used for the cultivation of RAW264.7. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$ and 95% air (incubator HERAcell 150i, Thermo Scientific).

*Blood Collection and Isolation of Neutrophils.* Human neutrophils were isolated from the blood (containing sodium citrate) of healthy volunteers who had given their informed consent. Volunteers were both female and male and aged between 18 and 60 years; they did not take any drugs and had been free of any signs of cold or other diseases for at least 3 weeks before blood collection. The Ethics Committee for Research at Masaryk University, Brno, Czech Republic (number EKV-2018-083) approved the collection protocol. The whole blood was mixed with 3% dextran and left at room temperature for ∼30 min. The resulting buffy coat was overlaid on a Histopaque 1077 instrument and centrifuged at 390g for 30 min without acceleration or brake. After centrifugation, the remaining erythrocytes were lysed by water hypotonic lysis. The neutrophils were washed in cold PBS, centrifuged at 190g for 10 min, and finally resuspended in cold PBS. The viability of neutrophils was verified with a CASY cytometer (Roche, Switzerland) and only neutrophils with viability exceeding 95% were used for experiments.

*Cytotoxicity Determination.* Cytotoxicity determination was performed according to the protocol of standard EN ISO 10993-5 (2019) with modifications. Cells were seeded in a concentration of 1 X $10^5$ cells $mL^{-1}$ and precultivated for 24 h. Then, the culture medium was replaced with colloids freshly diluted with medium to concentrations of 1, 2.5, 5.0, 10, 15, and 20% (**Table 1**).

**Table 1.** Concentration of PANI Colloids ($\mu$g $mL^{-1}$) Related to the Dilution (%) of the Parent Colloidal Dispersion

| % of parent colloidal dispersion used for cytotoxicity testing | concentration of PANI in colloids ($\mu$g $mL^{-1}$) | | |
|---|---|---|---|
| | PANI-CHIT | PANI-PVA | PANI-N |
| 100 | 791 | 308 | 754 |
| 20 | 158 | 61.6 | 150.8 |
| 15 | 118.7 | 46. 2 | 113.1 |
| 10 | 79.1 | 30. 8 | 75.4 |
| 5 | 39.6 | 15.4 | 37.7 |
| 2.5 | 19.8 | 7.7 | 18.9 |
| 1 | 7.9 | 3.1 | 7.5 |

In the case of RAW264.7, the cells were also activated by lipopolysaccharides (LPSs, 25 ng $mL^{-1}$). As a reference, cells cultivated in a medium without colloids were used. After 1 day of cultivation, the effects of colloids on cell viability were assessed by the MTT assay (Invitrogen Corporation). Absorption was measured at 570 nm using an Infinite M200 Pro NanoQuant instrument (Tecan, Switzerland).

The cell viability of the reference sample was set as 1 (100% viability). According to ISO 10993-5 (2019), a viability higher than 0.7 represents an absence of cytotoxicity, while samples with a viability lower than 0.7 are determined to be cytotoxic. The cell morphology was observed using an Olympus inverted fluorescence microscope (Olympus, CKX 41, Japan).

*Apoptosis of Neutrophils*. The apoptosis of neutrophils was determined with an Annexin V Apoptosis Kit-FITC (Exbio, Prague, Czech Republic). Neutrophils were incubated in a cytometric tube at a concentration of 2.5 X $10^5$ cells per tube and incubated with tested compounds (at concentrations of 1-15%) for 2 h. After exposure, the reaction mixture of annexin V (1:150 Annexin V-FITC and 1X Annexin V binding buffer) was added and incubated for 20 min in the dark; 2 $\mu$L of propidium iodide (PI, final concentration 1 mg $mL^{-1}$) was added to each sample and analyzed by flow cytometry (FACSVerse, BD Bioscience, Carlsbad, CA).

*Oxidative Burst of Isolated Neutrophils*. The oxidative burst of isolated neutrophils was measured by luminol-enhanced chemiluminescence (CL) using an LM-01 microplate luminometer (Immuno-tech, Czech Republic). The principle of the method was described previously.[24] Briefly, the reaction mixture for spontaneous (non-stimulated) CL consisted of 2.5 X $10^5$ isolated neutrophils per well, 1 mM luminol (10 mM stock solution in 0.2 mol $L^{-1}$ borate buffer), and the tested compounds (PANI-CHIT and PANI-PVa). Furthermore, for stimulated-CL, opsonized zymosan particles (OZP; 62.5 $\mu$g $mL^{-1}$) were used. Hank's balanced salt solution (HBSS) was used to adjust the total reaction volume to 250 $\mu$L. The CL activity of the samples was measured immediately at 37 °C and recorded continuously for 120 min. Data were converted to a percentage of the spontaneous or activated reference to achieve final results.

*Reactive Oxygen Species (ROS) Antioxidant Activity in a Luminol-HRP-H2O2 Cell-Free System*. The principle of the method was described previously.[25] As a source of CL signals, $H_2O_2$ and horseradish peroxidase (HRP) were used. Aliquots of 50 $\mu$L of PANI-CHIT or PANI-PVA (final concentrations 1-15%), HRP (final concentration, 2 U $mL^{-1}$), and luminol (final concentration, 10 $\mu$mol $L^{-1}$) were mixed in a 96-well luminescence plate. The reaction was initiated by adding $H_2O_2$ to a final concentration of 100 $\mu$mol $L^{-1}$. The CL signal was measured for 120 min at 37 °C with an LM-01 microplate luminometer (Immunotech, Czech Republic). Data were converted to a percentage of the control.

*Nitric Oxide (NO) Production by Murine Macrophages.* Changes in NO production were measured indirectly as the accumulation of nitrites (the end product of NO metabolism) in the medium using the Griess reagent (Sigma-Aldrich) according to the method described previously.26 RAW264.7 cells (0.5 X $10^5$ cells per well) were incubated in a 24-well plate with PANI-CHIT or PANI-PVA (final concentrations, 1-15%) and LPSs from Escherichia coli (25 ng $mL^{-1}$) for 24 h at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. LPS (25 ng $mL^{-1}$) alone was used as a positive reference. At the end of the incubation period, culture media were collected from the respective wells and centrifuged (16 000g, 4 °C, 5 min); 100 $\mu$L of centrifuged medium and the Griess reagent were mixed in a 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance was measured at 546 nm, using sodium nitrite (in the concentration range of 0-52 $\mu$mol $L^{-1}$) as the standard for the calibration curve. Data were converted to a percentage of the LPS control.

*IL-6 Production by Murine Macrophages.* The concentration of the proinflammatory cytokine interleukine-6 (IL-6) produced by RAW264.7 cells in the cultivation medium was determined after 24 h of exposure to PANI-CHIT or PANI-PVA (final concentrations, 115%) and LPS (25 ng $mL^{-1}$) by commercially available immunoassays (Mouse IL-6 DuoSet, R&D Systems). The assays were performed according to the manufacturer's instructions as described previously.[27] For more detailed information on culturing and preparing the medium, see the section: **"Nitric Oxide (NO) Production by Murine Macrophages".**

**Table 2.** Size ($z$-Average Diameter ± SD) and Polydispersity Index (PDI ± SD) of Colloidal Particles Stabilized with PVA or CHIT as the Steric Stabilizer[a]

| | before dialysis | | after dialysis | | after 24 months of storage | |
|---|---|---|---|---|---|---|
| sample | $z$-average (nm) | PDI | $z$-average (nm) | PDI | $z$-average (nm) | PDI |
| PANI-CHIT | 585 ± 10 | 0.41 ± 0.06 | 655 ± 7 | 0.45 ± 0.03 | 541 ± 2 | 0.30 ± 0.02 |
| PANI-PVA | 206 ± 2 | 0.50 ± 0.04 | 240 ± 2 | 0.55 ± 0.01 | 175 ± 3 | 0.52 ± 0.02 |
| PANI-N | 2800 ± 81 | 0.33 ± 0.05 | 3500 ± 74 | 0.33 ± 0.13 | n.d.[b] | n.d.[b] |

[a]Comparison with PANI prepared without a stabilizer. [b]Not determined.

*Statistical Analysis.* Data of cytocompatibility experiments are presented as the mean ± standard error of the mean (SEM). All assays were performed in quadruplicate. The number of independent experiments ($n$) is stated in the figure legend. The data from some of the measurements were normalized to the reference in each experiment to account for the variability of individual cell passages. Statistical analysis was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, CA. Statistical differences were tested by one-way ANOVA, which was followed by Dunnett's multiple comparison test or by a one-sample t-test to compare values expressed as percentages. In the case of the one-sample $t$-test application, the Bonferroni correction of the p-value for multiple comparisons was performed. $p < 0.05$ was taken to indicate significant differences between data mean values.

## RESULTS AND DISCUSSION

*Particle Size and Stability Are Controlled by the Type of Polymer Stabilizer*. The colloidal particles exhibited different sizes depending on the type of the polymer used for their stabilization **(Table 2)**, and PANI-PVA colloids were smaller in size (206 ± 2 nm) than samples prepared in the presence of chitosan, showing a $z$-average diameter of 585 ± 10 nm. The widths of distribution expressed as PDI were 0.55 and 0.45 for PANI-PVA and PANI-CHIT afer dialysis, respectively. After dialysis, conducted with the aim of removing residual impurities, which might compromise the biological tests, the average particle size in the samples increased. In our previous study, the average size of PANI colloidal particles stabilized with PVA and synthesized by the oxidative polymerization of aniline with ammonium persulfate (APS) was 116 nm (measured by DLS).[28] The particle size of PANI-CHIT samples from the enzyme-assisted process was also comparable with previously published sizes of colloidal PANI particles stabilized with chitosan and prepared by APS oxidation;[13] however, their morphologies were different. It is clear from the results that the size of colloidal PANI-CHIT particles obtained from the enzymatic reaction is well within the range of sizes prepared by the standard reaction employing APS for oxidation.

In contrast to PANI-PVA or PANI-CHIT, the freshly prepared sample without a stabilizer, PANI-N, was not of a true colloidal character, and particles greater than 3 $\mu$m sedimenting soon after dialysis were formed. Nevertheless, after homogenization, a uniform dispersion formed again, which was stable for several hours. If we compare enzymatically prepared PANI-N with PANI prepared by polymerization with APS, both without a stabilizing polymer, the nature of the enzymatically prepared sample was different and its colloidal stability was significantly higher. The explanation for this may lie in the stabilizing effect of peroxidase. Peroxidase is a protein (molecular weight of $\sim$44 X $10^3$ g $mol^{-1}$), which in fact can act as a polymeric stabilizer.[29] The resulting sample, therefore, exhibited different properties between standard PANI powder prepared in the absence of a stabilizer using APS and true colloidal particles prepared with the stabilizing effects of polymers of higher molecular weights.

The colloidal stability of PANI-PVA and PANI-CHIT was confirmed even after 24 months of storage at 5 ± 2 °C **(Table 2)**. For studied samples, the particle size was reduced by storage to 83% (PANI-CHIT) and 73% (PANI-PVA) of the initial values. This decrease was not surprising, as a corresponding effect was observed for CHIT-stabilized PANI colloids prepared by standard chemical oxidation.[13] A drop in the particle size during storage was also reported by Li et al.[30] for colloidal particles made of PVA-stabilized polypyrrole who suggested that this process may be driven by size changes in certain polymer agglomerates that occur during storage. Another option for the size reduction mentioned here could be time-induced changes in the polymer stabilizers used. The size distribution (expressed as PDI) of PANI-CHIT broadened after storage, but the distribution curve remained monomodal. On the contrary, the PDI value of PANI-PVA remained nearly unchanged after storage, but the initially monomodal distribution curve changed to a bimodal one, indicating the formation of two distinct particle populations.

*Antioxidation Activity Depends on the PANI Concentration in Colloidal Particles.* The antioxidation activity of polyaniline and its composites has previously been documented in several studies.[22,31,32] Also, the current study unambiguously confirmed the antioxidation activity of PANI-CHIT and PANI-PVA samples, which were both capable of scavenging DPPH radicals. Furthermore, the analyses revealed that the samples' activity differed. The efficacy of PANI-CHIT was higher immediately after mixing with DPPH*; however, with increasing time, the activity reduced and reached a plateau. The activity of PANI-PVA, on the other hand, was not as high as that for PANI-CHIT in short reaction times, but it gradually increased with a prolonged time of contact with radicals. The earlier onset of antioxidation activity for PANI-CHIT samples can be correlated with their higher concentration of PANI (791 $\mu$g $L^{-1}$) in comparison with PANI-PVA (308 $\mu$g $L^{-1}$). Differences in the course of PANI-PVA and PANI-CHIT scavenging activities might also be correlated with differences in their particle size. In summary, PANI-CHIT and PANI-PVA scavenging activities were 82 and 67% after 60 min, respectively. It should also be mentioned that after being exposed to ethanolic DPPH, the aqueous dispersions of PANI-CHIT and PANI-PVA gradually precipitated, forming particles on the bottom of the cuvette. This effect, however, did not compromise their antioxidation activity.

*Spectra of Colloidal Particles Demonstrate the Presence of the Conducting Emeraldine PANI Salt.* The process of the formation of polymer-stabilized PANI colloids and nonstabilized PANI was monitored by UV-vis spectroscopy. During synthesis, the reaction mixtures became blue. The color change confirmed the formation of polyaniline oligomers induced by hydrogen peroxide. This reaction step was very short, lasting for only 20 s, and was followed by a rapid change in the color of the reaction mixture to dark green, indicating thus the formation of the PANI emeraldine salt. An exception was the PANI-N sample prepared in the absence of a stabilizer, where the reaction mixture did not turn dark green but brown. The UV-vis spectra of all samples (PANI-PVA, PANI-CHIT, PANI-N) recorded in two different dilution media (1 mol $L^{-1}$ HCl and water) within a wavelength range from 300 to 800 nm are given in

**Figure 1.** The UV-vis spectra of the PANI-PVA colloid were similar in both media and showed a broad peak at 400 nm and a peak with a maximum at about 750 nm.

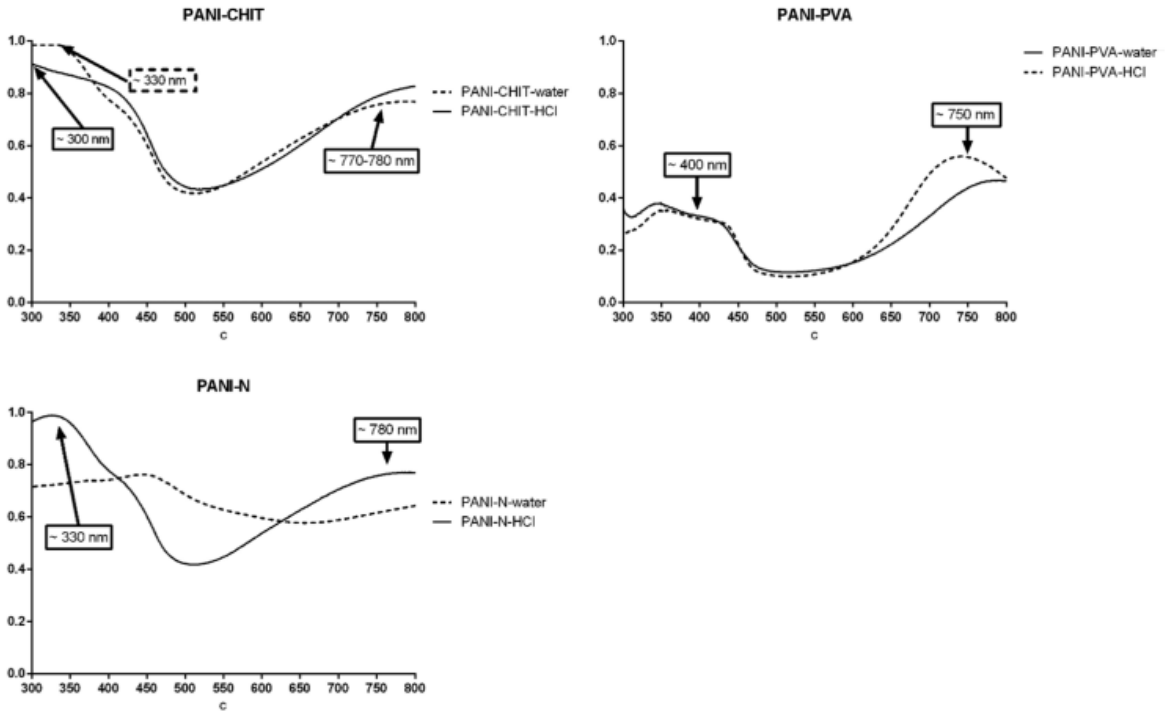


**Figure 1.** UV-vis spectra of enzymatically synthesized PANI stabilized with PVA and CHIT or without a stabilizer (PANI-N).

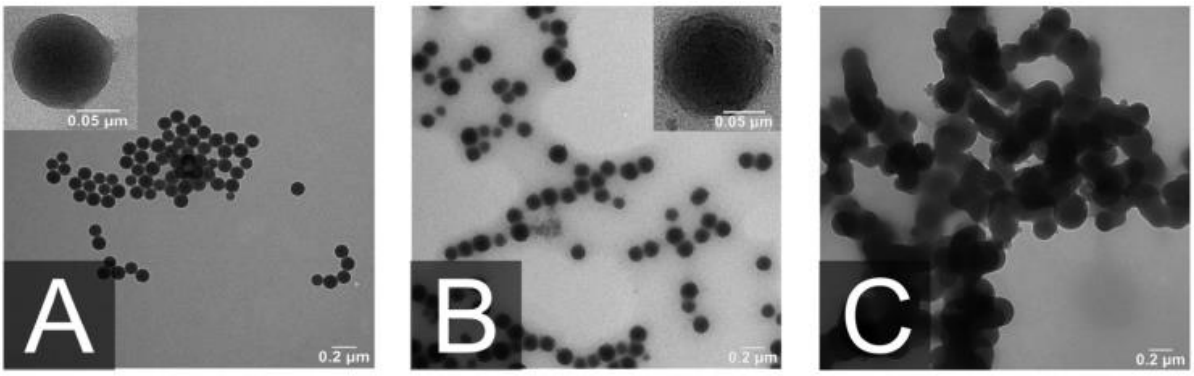


**Figure 2.** Transmission electron micrographs of PANI-CHIT (A), PANI-PVA (B), and PANI-N (C) colloids.

The peak at ~400 nm arose from the $\pi - \pi *$ electron transition within the benzenoid ring and the peak at ~750 nm evidenced the formation of a polaron structure. The $\lambda_{max}$ of the latter peak shifted to 740 nm for PANI-PVA measured in HCl in comparison with spectra in water, indicating differences in polaron delocalization. When measured with the acidity of the medium fixed, i.e., in 1 mol L-1 HCl, the maximum absorbance in the $\lambda$ range from 350 to 430 nm may be taken as an approximate measure of the concentration of PANI in the dispersion. The spectrum of PANI-CHIT colloids shows maxima at $\lambda$ of ~330 and 770 nm in water, and 300 and 780 nm in HCl. In the study,[12] PANI colloids stabilized with chitosan prepared by standard chemical oxidation with APS showed a similar course of spectra, with slightly shifted distinct maxima at 390 and 800 nm. The abovementioned difference in the color

between nonstabilized PANI-N (brown) and PANI-PVA and PANI-CHIT (green) is also evident from the spectra obtained on the PANI-N sample dispersed in water, which does not show a course typical of the green PANI salt. After the dispersion medium was changed to 1 mol $L^{-1}$ hydrochloric acid, the spectra converted to their common course **(Figure 1)**. The reason for this is obvious as experimental data published in ref **33** show that the oxidation products of PANI formed in neutral and weakly acidic media are brown in color with low conductivity but turn to the green emeraldine salt in a more strongly acidic environment.

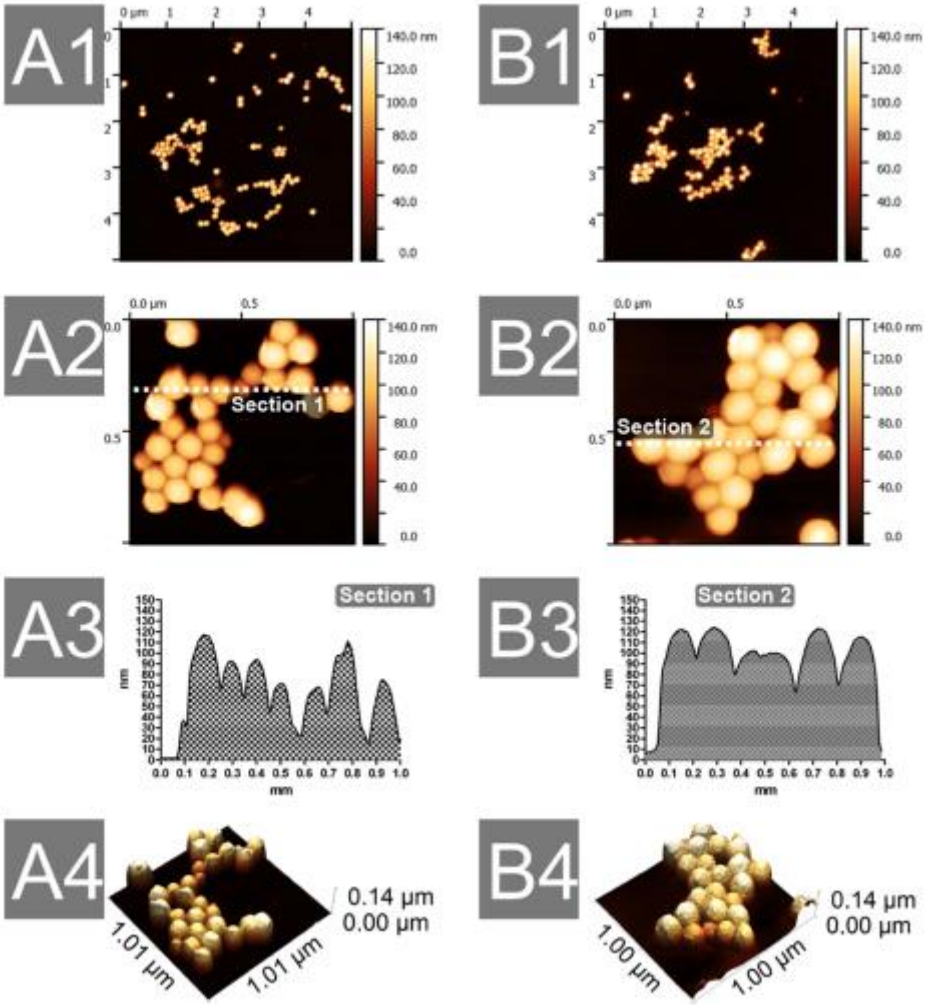


**Figure 3.** Colloidal particles on mica surfaces characterized by AFM. Height images and profiles of PANI-CHIT (left) and PANI-PVA (right). PANI-N formed aggregate-like structures and, thus, are not presented.

*Morphology of Colloidal Particles—Stabilizers Are a Key Factor in the Formation of Colloidal Particles.* The morphology, size, and shape of the prepared PANI colloids were depicted using TEM **(Figure 2)**. The microscopy observations revealed regular, spherical particles of similar sizes, with relatively narrow distributions for both PVA- and CHIT-stabilized samples. The average diameters estimated by TEM were 126 ± 9 and 133 ± 13 nm for PANI-CHIT and PANI-PVA, respectively. In contrast, PANI-N synthesized without a stabilizing agent was formed by particles, which were obviously bigger than those present in polymer-stabilized samples and exhibited significant aggregation caused by the absence of a stabilizing polymer. The TEM analyses roughly complied with results from the scattering

measurements. However, differences between the sizes of PVA- and CHIT-stabilized samples measured by DLS were bigger in comparison with TEM measurements, thanks to the different measuring principles of the DLS technique. Bigger PANI-CHIT particles can originate from the higher molecular weight of CHIT (310 000-375 000 g $mol^{-1}$) compared to PVA (89 000-98 000 g $mol^{-1}$) stabilizers, which are detected as loose layers around the core particles by DLS but are not visible after particle drying for TEM analysis. Compared to other standard PANI-based colloids, enzymatically polymerized colloids with the CHIT or PVA stabilizer are spherically symmetrical. After deposition on a solid substrate for TEM or AFM imaging, they do not disintegrate into the randomly arranged elliptical or fibrillar structures discussed in[13] and some other papers.[16,17]

Atomic force microscopy (AFM) confirmed the spherical shapes of PANI-PVA and PANI-CHIT particles on the mica surface. In contrast, the AFM analysis of PANI-N was not feasible, as the size of the particles/agglomerates were unsuitable for such analysis. The particles stabilized by CHIT were characterized by diameters ranging from 50 to 110 nm and heights ranging from 60 to 120 nm. Colloidal particles stabilized by PVA exhibited diameters from 90 to 150 nm and heights from 100 to 130 nm. Nonstabilized PANI-N formed aggregate-like structures with an average width and height in the order of units of micrometers on the mica surfaces. The resulting sizes from AFM differed from those measured by DLS analysis. However, they fully comply with TEM **(Figure 3).**

*Cytotoxicity Is Related to the Concentration of PANI.*Cytotoxicity is one of the basic biological properties determining the applicability of materials. Here, cytotoxicity was evaluated using two different cell lines, fibroblasts (NIH/ 3T3) and macrophages (RAW264.7). As these cell lines differ in their phenotypes and properties (fibroblasts are the most common cells of soft tissues and are mostly used for cytotoxicity evaluation, while macrophages are immune cells, which are moreover activated by LPS before testing), the results presented here are widely representative.

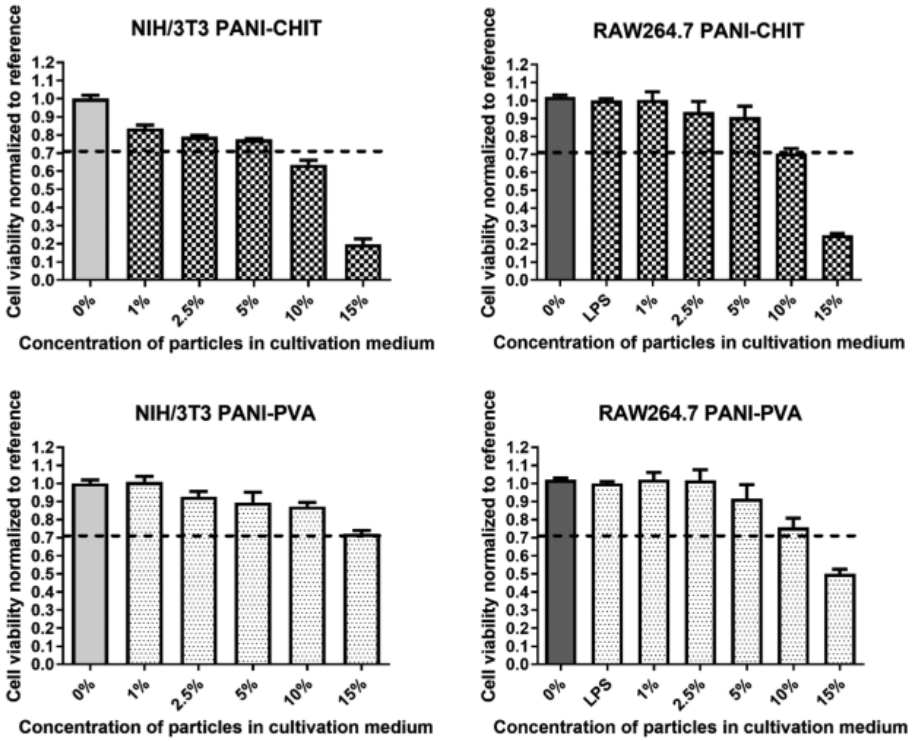


**Figure 4.** Cell viability in the presence of different concentrations of colloidal particles in cultivation medium. Data were converted to a relative value of the reference and expressed as the mean ± SEM ($n$ = 3). According to EN ISO 10993-5, samples were deemed cytotoxic if the cell viability fell below a threshold value of 0.7.

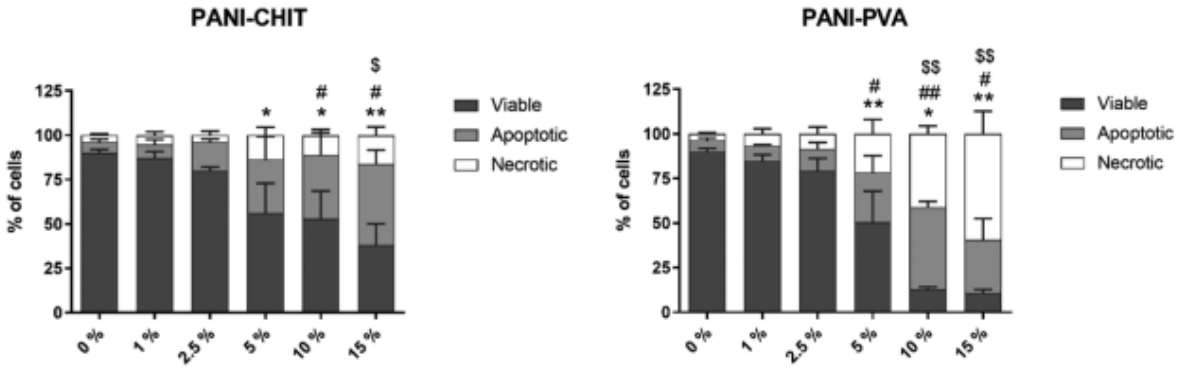


**Figure 5.** Effect of PANI-PVA and PANI-CHIT (1-15%) on the induction of apoptosis/necrosis in isolated neutrophils. The distribution of viable, apoptotic, and necrotic cells was determined on the basis of annexin V/PI staining after 2 h. Data were expressed as the mean ± SEM ($n$ = 3) and analyzed by ANOVA combined with Dunnett's test ($^{**/\#\#/\$\$}p < 0.01$ and $^{*/\#/\$}p < 0.05$). * represents the statistical analysis of the viable cells, # represents the statistical analysis of the apoptotic cells, and $ represents the statistical analysis of the dead cells.

For isolated and short-lived neutrophils (viable for 6-12 h), we determined the percentage of viable, apoptotic, and necrotic cells to distinguish the reason for cytotoxicity **(Figure 5).**

As can be seen, the cytotoxic effect was found to be very similar in the case of both cell lines **(see Figures 4 and 5)**. The noncytotoxic threshold was achieved at dilutions of the colloidal dispersion in the cultivation medium lower than 10%, except for PANI-CHIT on NIH/3T3 cell lines. Here, cell viability slightly decreased below the noncytotoxic threshold at 10% sample dilution. In the more sensitive isolated neutrophils, the ratio of apoptotic and necrotic cells increased compared to live cells. At 5% of PANI-PVA, neutrophil viability was reduced to about 50% of the total cells, and at 10 and 15% of PANI-PVA, the viability was reduced to 15% of the total cells. In 5 and 10% of PANI-CHIT, neutrophil viability decreased to ~50% of the total cells, and in 15% of PANI-CHIT, the viability decreased to ~35% of the total cells. The cytotoxic limits of the colloidal dispersions tested here obviously correlate with the contents of PANI.

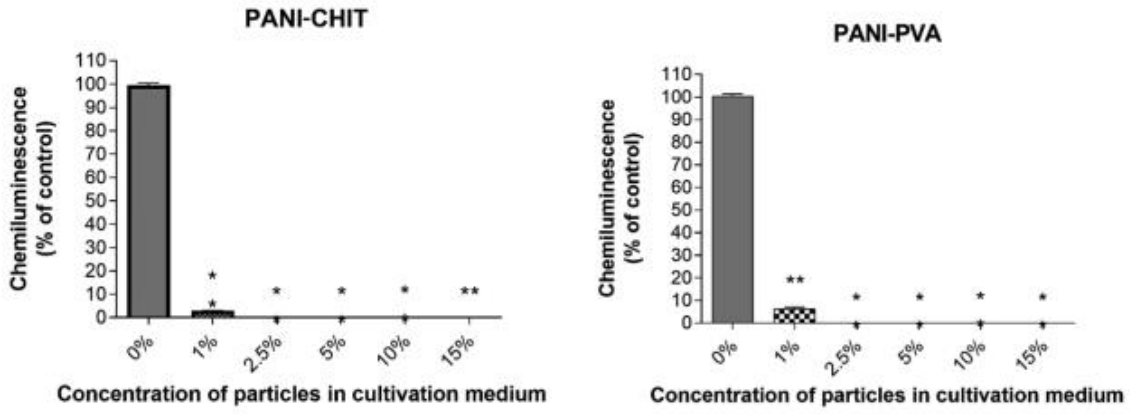


**Figure 6.** Antioxidant activity of PANI-PVA and PANI-CHIT. Data were converted to a percentage of the control and expressed as the mean ± SEM ($n$ = 6). One-sample $t$-test was used to analyze the significance of the obtained data separately comparing the effect of each compound with the control. A Bonferroni correction of the p-value for multiple comparisons was performed ($** p < 0.01$).

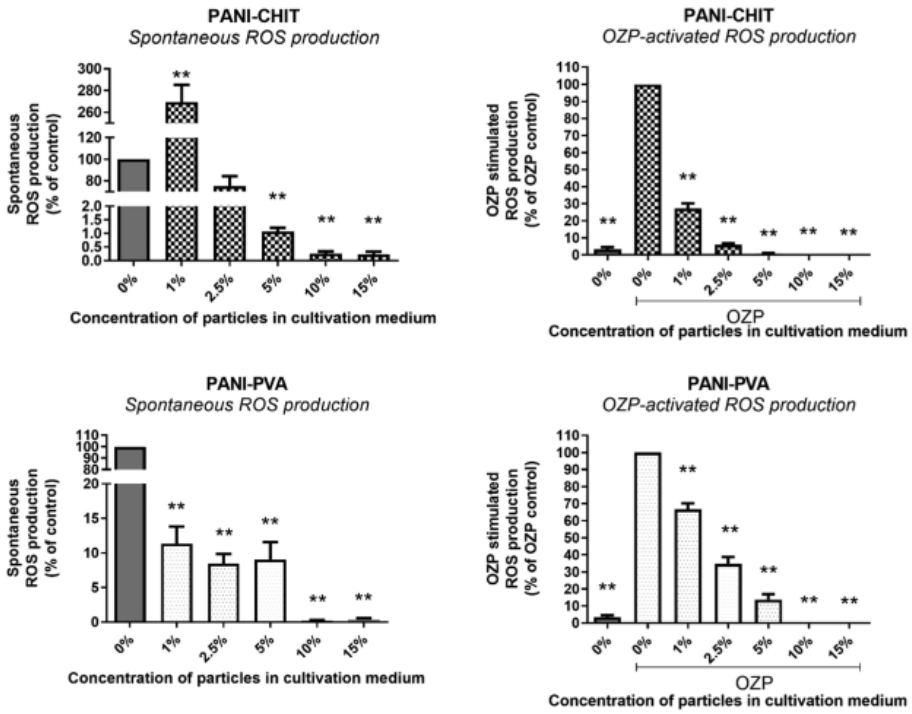


**Figure 7.** Effect of colloidal particles on spontaneous ROS production (left) and on OZP-activated ROS production (right) by neutrophils. Data were converted to a percentage of the control and expressed as the mean ± SEM ($n$ = 4). One-sample $t$-test was used to analyze the significance of the obtained data separately comparing the effect of each compound with the control. A Bonferroni correction of the $p$-value for multiple comparisons was performed ($** p < 0.01$).

It has previously been reported that the cytotoxicity of PANI-based colloids could be affected not only by the concentration of PANI but also by the type of stabilizer. While the PVA stabilizer is commonly considered biocompatible,[34] the biocompatibility of CHIT is still controversial. The potential cytotoxic effect of CHIT particles was also shown to depend on the particle size[35] and could also be induced by the molecular weight of the stabilizing chitosan.[36] Kasparkova et al.[13] recently investigated the biocompatibility of PANI-CHIT colloids, and their results somewhat differ from those obtained in the current study. The absence of cytotoxicity (viability >0.8 in comparison with the reference, determined according to ISO 10993-5/2009) was observed up to a higher concentration of 90 $\mu$g mL$^{-1}$ PANI in colloidal particles, while in the current work, the noncytotoxic threshold was 80 $\mu$g mL$^{-1}$ PANI in colloidal particles. In this case, however, the protocol of ISO 10993-5 followed new limits (viability >0.7 in comparison with the reference, as given by the standard version from 2019). Based on the observations by Kasparkova et al.,[13] it can be concluded that enzymatically prepared samples are somewhat more cytotoxic in comparison with samples prepared by standard chemical oxidation.

*Antioxidant Activity Contributes to Immunomodulatory Properties of Colloidal Dispersions*. The immunomodulatory activity of PANI-PVA and PANI-CHIT was studied using two types of selected professional phagocytes, neutrophils (acting in blood circulation) and macrophages (acting in the tissues). The activity of both immune cell types is closely connected to the production of reactive oxygen (ROS) and nitrogen (RNS) species. Both types of phagocytic cells form an essential part of the innate immune system.

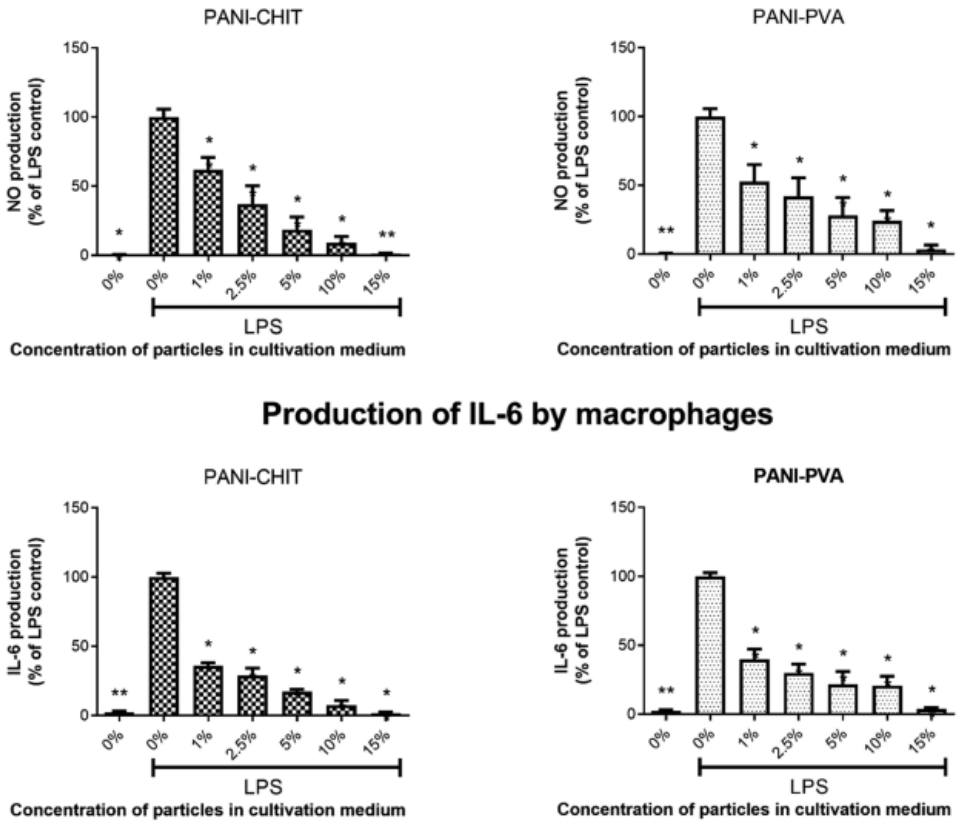


**Figure 8.** Effect of PANI-PVA and PANI-CHIT on NO and IL-6 production by RAW264.7 macrophages. Data were converted to a percentage of the LPS reference and expressed as the mean ± SEM ($n$ = 3). One-sample f-test was used to analyze the significance of the obtained data separately comparing the effect of each compound with the reference. A Bonferroni correction of the p-value for multiple comparisons was performed ($** \; p < 0.01$).

The antioxidant activity of tested compounds plays an important role in reducing the immune response and the CL signal. Therefore, we focused on detecting the antioxidant activity of PANI-PVA and PANI-CHIT.

As presented in **Figure 6**, both types of colloidal particles express high antioxidant activity. Even at a 1% dilution of PANI-PVA (a PANI concentration of 3.1 $\mu$g mL$^{-1}$) and PANI-CHIT (a PANI concentration of 7.9 $\mu$g mL$^{-1}$), the colloidal particles showed a significant reduction to 6.45 and 2.85% of the reference, respectively. The other tested concentrations of PANI-PVA and PANI-CHIT decreased the CL signal to 0% of the reference **(Figure 5)**.

*Colloidal Particles Significantly Reduced ROS Production in Neutrophils*. First, the effect of the presence of colloidal particles on the detectable amount of ROS produced by neutrophils spontaneously and after their activation was tested.

Both types of colloidal particles significantly reduced the amount of detectable ROS produced by neutrophils spontaneously or after their activation **(Figure 7)**. PANI-PVA significantly reduced ROS production in all tested concentrations. In contrast, 1% PANI-CHIT significantly upregulated spontaneous ROS production at other tested concentrations of PANI-CHIT, and the ROS production was reduced. At 5% dilutions of all tested samples (a PANI concentration of 15.4 $\mu$g mL$^{-1}$ in PANI-PVA and 39.6 $\mu$g mL$^{-1}$ in PANI-CHIT), spontaneous ROS production was reduced. When OZP was used as the activator, the tested colloidal particles were able to significantly reduce ROS production in a dose-

dependent manner (**Figure 6**). Considering the antioxidant activity of colloidal particles (**Figure 5**), the effect is related to the inactivation of ROS, not to the interaction between colloidal particles and neutrophils itself.

*Colloidal Particles Significantly Reduced NO and IL-6 Production in Macrophages.* After their activation, for example, by bacteria or parts thereof, such as LPS, many proinflammatory compounds are produced by macrophages (e.g., cytokines, chemokines, or NO). Here, the two important proinflammatory markers of macrophage activation were detected—namely, NO and IL-6.

Both tested types of colloidal particles were able to significantly reduce the production of NO and IL-6 in a dose-dependent manner (**Figure 8**). When compared to their potential cytotoxic activity at higher concentrations, the tested colloidal particles showed similar anti-inflammatory activity. From **Figures 5-7**, we can conclude that colloidal particles exhibit an anti-inflammatory effect related to their antioxidant activity, especially in the case of neutrophils.

## CONCLUSIONS

Biocompatible polymers, PVA or chitosan, were used as steric stabilizers during the enzymatic polymerization of aniline in dispersed media to create PANI colloidal particles. A sample without a stabilizing component was prepared for comparison. All tested colloidal particles showed in vitro antioxidation activity determined via scavenging of DPPH radicals. Cytotoxicity testing demonstrated that all colloids exhibited low toxicity. Samples up to concentrations of 31 $\mu$g mL$^{-1}$ (PANI-PVA) and 40 $\mu$g mL$^{-1}$ (PANI-CHIT) did not show cytotoxicity (the cell viability was higher than 70%). The cytotoxicity depended mainly on the polyaniline concentration and somewhat on the type of stabilizer. All tested colloidal particles reduced oxidative stress and hindered the release of inflammatory cytokines by macrophages. The observed antioxidant capacity can be especially advantageous in the early stages of inflammation. The original approach to synthesis used here, employing environmentally friendly and biocompatible horseradish peroxidase, demonstrates a smart way of preparing conducting particles with unique properties, which can be further modified by the stabilizers used. Overall, the results of this study indicate that colloidal particles based on conducting polyaniline and chitosan (or PVA) produced by enzymatic synthesis are promising systems for biological applications.

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