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Colloidal polyaniline dispersions: Antibacterial activity, cytotoxicity and neutrophil oxidative burst

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ABSTRACT

Polyaniline colloids rank among promising application forms of this conducting polymer. Cytotoxicity, antibacterial activity, and neutrophil oxidative burst tests were performed on cells treated with colloidal polyaniline dispersions. The antibacterial effect of colloidal polyaniline against gram-positive and gram-negative bacteria was most pronounced for *Bacillus cereus* and *Escherichia coli*, with a minimum inhibitory concentration of 3500 $\mu g\,mL^{-1}$. The data recorded on human keratinocyte (HaCaT) and a mouse embryonic fibroblast (NIH/3T3) cell lines using an MTT assay and flow cytometry indicated a concentration-dependent cytotoxicity of colloid, with the absence of cytotoxic effect at around 150 $\mu g\,mL^{-1}$. The neutrophil oxidative burst test then showed that colloidal polyaniline, in concentrations <150 $\mu g\,mL^{-1}$, was not able to stimulate the production of reactive oxygen species in neutrophils and whole human blood. However, it worked efficiently as a scavenger of those already formed.

1. Introduction

Ever increasing numbers of papers dealing with the biocompatibility of conducting polymers are evidence of the growing interest in these materials. Whereas polypyrrole is better described and more intensively studied in the context of biological properties, information about polyaniline uses in biosciences is less frequent. So far, conductivity and electrochemical behavior were the main focuses of attention. The interesting properties of polyaniline have recently led to an investigation of its possible uses in biomedical applications. This concerns especially the objects that are associated with electrical properties, such as brain, cardiac or neural tissues and cells. Namely, applications in cardiomyocyte synchronization [1], myoblast differentiation [2], neuronal lineage differentiation [3], skeletal muscle [4] or cardiac tissue engineering [5] have been reported. Also, the cytotoxicity, irritation and sensitization potential [6], cell proliferation [7] and antibacterial properties [8–10] of polyaniline powder prepared according to a procedure provided by IUPAC [11] have already been described. Other studies, dealing with biological properties, such as the *in-vivo* tissue response of polyaniline, are mostly based on the testing of polyaniline films cast on various carrier surfaces [12], polyaniline composites [13] or electrospun blends [14].

Although the biological applications of polyaniline are on the rise, they are limited to a certain extent by its insolubility in aqueous media. It is commonly known that conducting polyaniline is poorly soluble even in organic solvents, which strongly influences its processability. Hence, a considerable effort has been devoted to the preparation of processable forms of this polymer. Possible solutions to this challenge can be found in copolymerization [15] or protonation with acids containing relatively long alkyl side chains, which may enhance the solubility in solvents [16]. The preparation of conducting polymer colloids is another approach how to cope with this problem.

Colloidal polyaniline dispersions are prepared when aniline is oxidized in an aqueous medium containing a suitable water-soluble polymer acting as a steric stabilizer. Various polymers have been tested as stabilizers, including poly(vinyl alcohol-*co*-vinyl acetate) [17], poly(vinyl alcohol) [18], poly(methyl vinyl ether) [19], poly(ethylene oxide) [20] or cellulose ethers [21–23]. Also poly(*N*-vinylpyrrolidone) turned out to be an efficient stabilizer of polyaniline colloidal particles and has been successfully employed [24–26]. In light of the numerous applications of colloidal

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polyaniline that have been proposed [27–29], it seems worthwhile to examine the basic biological properties of this promising material.

In the present study, a colloidal polyaniline dispersion, employing poly(*N*-vinylpyrrolidone) as the stabilizer, was prepared and characterized. The main target of the study was to determine the influence of the colloid on prokaryotic and eukaryotic cells via assessing the cytotoxicity on two cell lines and determining the antibacterial properties on representatives of gram-positive and gram-negative bacteria. In addition, the generation of human reactive oxygen species by neutrophils has also been studied. The biological properties of colloidal polyaniline are being reported here for the first time.

2. Materials and methods

2.1. Preparation of colloidal polyaniline dispersion

Aniline hydrochloride (0.2 M) was oxidized with ammonium peroxydisulfate (0.25 M) [30] in the presence of a stabilizer, 2 wt% poly (*N*-vinylpyrrolidone) (PVP; Fluka, type K90, molecular weight 360,000). Aniline hydrochloride (259 mg) was dissolved in an aqueous solution of PVP (4 wt%) to 5 mL of solution. The polymerization of aniline was started at room temperature, close to 20 °C, by adding 5 mL of aqueous solution containing 571 mg ammonium peroxydisulfate. The mixture was briefly stirred and left at rest for 2 h. The resulting dark green dispersion of polyaniline hydrochloride was transferred into a membrane tubing (Spectra/Por 1, Spectrum Medical Instruments, USA; molecular weight cut-off 7,000) and exhaustively dialyzed against 0.2 M hydrochloric acid to remove residual monomers and by-products, such as ammonium sulfate.

2.2. Particle size

The size and distribution of the colloidal particles were determined by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). Measurements of the hydrodynamic radii of colloidal particles, expressed as z-average particle diameters, were performed at 25 °C. The intensity of scattered light ($\lambda = 633$ nm) was observed at a scattering angle of 173°. The polydispersity index (PDI) was evaluated by assuming log-normal distribution of particle sizes. Prior to measurements, the performance of the instrument was verified by using polystyrene latex nanoparticles with the nominal size of 92 ± 3 nm (Thermo Scientific, Germany).

2.3. Polyaniline concentration

Optical spectra of polyaniline colloids were recorded in the wavelength range of 200–800 nm with a Photo Lab 6600 UV-vis spectrometer (WTW, Germany) after defined dilution with 1 M hydrochloric acid. The concentration of polyaniline in colloidal dispersion was calculated from the absorbance at wavelength of 395 nm by using the Lambert–Beer law, $A = \varepsilon cl$, where $\varepsilon = 31{,}500 \pm 1{,}700\ \mathrm{cm^2\ g^{-1}\ cm^{-1}}$ is absorption coefficient [18], c is the concentration of polyaniline and $l = 1$ cm is the optical path.

2.4. Antibacterial testing

The testing of antibacterial properties of polyaniline was conducted with representatives of gram-negative and gram-positive bacterial strains. *Bacillus cereus* (CCM 2010), *Staphylococcus aureus* (CCM 3953), *Escherichia coli* (CCM 3954) and *Pseudomonas aeruginosa* (CCCM 3955) were employed in the test. All strains were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). Bacteria were grown on nutrient agar ($5\ \mathrm{g\ L^{-1}}$ peptone, $3\ \mathrm{g\ L^{-1}}$ beef extract, $15\ \mathrm{g\ L^{-1}}$ agar; Hi-Media Laboratories, India) and $5\ \mathrm{g\ L^{-1}}$ sodium chloride (Lach-Ner, Czech Republic) at 37 °C. Initial inocula of the microorganisms were prepared from the 24 h cultures, and bacterial suspensions were adjusted to contain 10^6 CFU $\mathrm{mL^{-1}}$ by diluting them with a nutrient broth containing $5\ \mathrm{g\ L^{-1}}$ peptone, $3\ \mathrm{g\ L^{-1}}$ beef extract and $5\ \mathrm{g\ L^{-1}}$ sodium chloride.

For antibacterial testing, the colloidal polyaniline dispersion was diluted with a nutrient broth to obtain polyaniline concentrations ranging from 2,000 to 8,500 $\mu\mathrm{g\ mL^{-1}}$ and inoculated with 200 μL of bacterial suspension. After a 24 h incubation, 100 μL of decimal dilutions were spread over agar plate surfaces and incubated for 24 h at 37 °C. Colonies were counted and minimum inhibitory concentration (MIC) was calculated. Each experiment was repeated four times.

The pH determination of the bacterial suspension in the absence and the presence of a polyaniline colloid was carried out by using a Spear pH meter tester (Eutech) before and after cultivation in order to monitor the pH changes occurring in the suspension during bacterial growth.

2.5. Test of cytotoxicity

Prior to *in-vitro* cytotoxicity testing, the samples were disinfected by dry heat at 120 °C for 40 min. Cytotoxicity testing was performed with a human immortalized keratinocyte cell line (HaCaT, Cell Lines Service, Catalog No. 300493, Germany) [31] and a mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3, USA). The HaCaT cells were cultivated using Dulbecco's Modified Eagle Medium – high glucose, with added 10% fetal bovine serum and penicillin/streptomycin, $100\ \mathrm{U\ mL^{-1}}$ (PAA Laboratories GmbH, Austria). The ATCC-formulated Dulbecco's Modified Eagle's Medium (catalog No. 30-2002), with added bovine calf serum to a final concentration of 10% and penicillin/streptomycin, $100\ \mathrm{U\ mL^{-1}}$, was used as the culture medium in case of NIH/3T3 cells.

The tested samples were diluted to concentrations of 520, 345, 171, 155, 130, 105, 70, 35, and 20 $\mu\mathrm{g\ mL^{-1}}$ in the culture medium. Cytotoxicity testing was conducted according to the EN ISO 10993-5 standard procedure, with modification. Cells were pre-cultivated for 24 h, and the culture medium was subsequently replaced with dilutions of polyaniline colloid. As a reference, cultivation in a pure medium without colloid was used. To assess cytotoxic effect, a MTT assay (Invitrogen Corporation, USA) was performed after one-day cell cultivation in the presence of colloidal polyaniline. All the tests were performed in quadruplicates. The absorption was measured at 570 nm with an Infinite M200 Pro NanoQuant (Tecan, Switzerland). Dixon's Q test was used to remove outlying values, and mean values were calculated. The cell viability is presented in two ways in order to provide a comprehensive view of the results: (1) as a percentage of cells present in the respective extract relative to cells cultivated in a pure extraction medium without colloidal polyaniline (100% viability), and (2) by using the *t*-test expressing the statistical differences between the averages of individual dilutions compared to the reference.

The morphology of the cells was assessed after 24 h of cultivation in the presence of a colloid. The changes in the cell morphology were observed with an Olympus inverted fluorescent microscope (Olympus, CKX 41, Japan). The fluorescent staining of DNA was performed using the Hoechst 33258 dye (Invitrogen Corporation, USA).

2.6. Apoptosis versus necrosis rate

To distinguish healthy, apoptotic and necrotic cells after the contact of the cell cultures with colloidal polyaniline, staining with annexin V/propidium iodide (BD Biosciences, Canada) was