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In vitro bio-immunological and cytotoxicity studies of poly(2-oxazolines)

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Abstract Poly(2-oxazolines) with varying alkyl chain lengths (e.g., methyl, ethyl, aryl) and molar masses have been tested for cell cytotoxicity in vitro. A standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the estimation of cell viability. Two monomers, 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline, were found to provide polymers with non-cytotoxic properties. The dependence of cell viability on molar mass confirmed the expected trend; the viability increased with the higher molar mass of poly(2-ethyl-2-oxazoline) (PETOX), up to 15,000 g/mol. The results obtained for the polymers with aliphatic side chains were compared with the analogues that possessed an aromatic moiety. All results confirmed low cytotoxicity of the polymers prepared by cationic polymerization of 2-alkyl- and 2-aryl-2-oxazolines, which supports their utilization in biomedical applications. Fluorescence microscopy and steady-state fluorescence were used to observe pyrene-labeled polymer interactions with living cells. Polymer accumulated within the cells was found to be dependent on polymer concentration in media. The immunoefficiency of aromatic and aliphatic oxazoline polymers and copolymers was also studied. Phagocytic and metabolic activities of macrophages were used to assess the immunosuppressive effects of the selected copolymers for possible applications in drug delivery and immunobiology. Overall, the tested polymers demonstrated no significant influences on the cellular immunological parameters.

1 Introduction

Synthetic polymers are considered promising materials for biomedical applications. Various polymer formulations have been employed to achieve the desired chemical, physical and biological properties. Recently, there has been much interest in the development of environmentally responsive polymers for use as biomaterials [1]. Such behavior is significant for the controlled release of drugs upon the application of a stimulus, such as pH, temperature, light or ionic strength.

These mentioned properties are necessary for the utilization of polymeric materials for biomedical applications, such as drug and gene delivery, biomembrane technology and biocatalysis [2, 3]. The designed polymers should also meet the requirements for biocompatibility and cytotoxicity. Biocompatibility assessment of a polymeric material includes adequate testing for undesired responses. To evaluate biocompatibility, examinations of acute and system toxicity, tissue cultures, cell growth inhibition, mutagenicity, carcinogenicity, teratogenicity and allergenic potential should be conducted [4]. In vitro cytotoxicity can be tested by a number of methods that are mostly based on the colorimetric assay of dyes that are sensitive to viable or dead

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cells. Such assays include 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), water soluble tetrazolium salts (WST), trypan blue or sulforhodamine B as dyes.

In addition to acute cytotoxicity, the study of humoral and cellular immunity is very significant to the biocompatibility assessment of polymeric materials. Macrophages and macrophage-like cells, which are ancient and phylogenetically-conserved cells, represent the first line of host defense, after the epithelial barrier, against foreign invaders that reach mucosal surfaces. These cells are able to immediately defend as well as send signals for the cell recruitment and antigen presentation to immune-competent cells. Therefore, these cells represent a critical component of the innate and specific immune responses to diverse challenges. This unique phagocytosis process is a hallmark of macrophage activity [5–7].

From a variety of polymers suitable for biological and medical use, 2-oxazoline-based polymers serve a significant role. Polymers prepared from 2-alkyl-2-oxazolines with a shorter alkyl chain are water-soluble, stimuli-sensitive and promise the use in drug and gene delivery, membrane technology and catalysis [8]. The important feature of poly(2-oxazolines) is also their tertiary amide structure which ensures the biocompatibility and suppressing the biodegradation compared to peptides [8].

Poly(2-oxazolines), or more accurately poly(*N*-acyl ethylene imines), can be prepared by living cationic polymerization initiated by various electrophilic species, including alkyl halogenides, sulfonic esters and strong mineral acids [9]. Resultant polymers are prepared in a controlled manner over its defined size, topology and required properties. Furthermore, these materials can be used in the synthesis of complex polymer systems, such as block- and graft-copolymers [10] or polymers with star- [11, 12] or comb-like architecture [13], which are suitable nanostructured materials and can be used for different applications. Poly(2-ethyl-2-oxazoline) (PETOX)represents a thermoresponsive polymer with the lower critical solution temperature (LCST) equal to 69°C [14]. Poly(2-isopropyl-2-oxazoline) also exhibits thermosensitive behavior, and its LCST is near 37°C, which is necessary for its utilization in drug and gene delivery [15, 16]. These materials can also be used as alternatives to poly(ethylene glycol) (PEG) for conjugation with proteins, saccharides or drugs [17]. Recently, 2-oxazoline based polymers have been used for the preparation of multivalent vaccines by the attachment of polysaccharide antigens to poly(2-oxazoline) backbone [18, 19]. From the point of described bioconjugations, the advantage of poly(2-alkyl-2-oxazolines) over PEG consists in the possible incorporation of functional groups necessary for attachment of bioactive molecules to the backbone or carrier polymer.

Although the promising utilization of 2-oxazoline polymers in biomedical applications has been described [8], little information is available about their biocompatibility and cytotoxicity. Park and coworkers [20] studied the cytotoxicity of PETOX as a function of hydrolysis to linear poly(ethylene imine) (PEI) by conducting an MTT assay. They found that cell viability decreased with an increasing degree of hydrolysis, and above 50% hydrolysis, the cell viability decreased to 20%. Partially hydrolyzed PETOX were used as DNA transfection agents. Another example of this polymer as a suitable material for medical use is employing polymeric micelles of poly(2-ethyl-2-oxazoline)-block-poly(ε-caprolactone) copolymer (PEtOz-PCLs) as a carrier for paclitaxel [21]. It was showed that the viability of KB human epidermoid carcinoma cells in the presence of PEtOz-PCL copolymers were in the range 80–100% depending on the concentration of the copolymer solution and also on the ratio of both building blocks. As seen from available results, poly(2-alkyl-2-oxazolines) are considered as polymer materials with relatively low cytotoxicity. However, the influence of their structure on cytotoxicity parameters has not been studied yet.

The intracellular applications of polymers require investigating the intracellular metabolic pathways of distribution of polymer in cells, which can be examined by employing labeled, polymeric systems. One possibility is labeling poly(2-oxazolines) with a fluorescent probe, which can be consequently analyzed with a confocal laser-scanning microscope (CLSM) [22]. Biodistribution, targeting and excretion can also be investigated by radiolabeled poly(2-alkyl-2-oxazolines). Authors used ^{111}In-labeled polymers to study the distribution and excretion of such polymers in mice [23]. Similarly, ^{125}I-labeled 2-oxazoline-based copolymers were examined after their intravenous administration to mice, and their biodistribution was assessed [24].

This work reports the cytotoxicity studies of poly (2-oxazolines) and the influence of structure and composition on their biocompatibility. Polymers bearing an end-caped fluorescent probe were used for imaging polymer accumulation in cells. Beside the study also evaluated and compared the influence of different 2-oxazoline-based polymers on macrophage cell viability and functionality. The following relevant immunobiological assays were conducted: (i) determination of phagocytic activity, accompanied by an oxidative burst and (ii) cell viability. Conducting functional studies on cell viability and the phagocytic and metabolic activities of macrophages represents a quantitative approach to consider the potential cytotoxic and/or immunosuppressive effects of exogenous substances and their interactions with macrophage cells.

2 Experimental part

2.1 Materials

2-Ethyl-2-oxazoline and 2-methyl-2-oxazoline (both Sigma-Aldrich) were dried over KOH for 48 h and distilled over calcium hydride (CaH_2) under low pressure. *N*,*N*-dimethylacetamide was stirred with KOH overnight and then distilled under vacuum over CaH_2. The initiators, methyl *p*-nitrobenzenesulfonate and 1-(bromoacetyl)pyrene, were purchased from Sigma-Aldrich and used as received. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO, both Merck, Germany) were used as received.

Phosphate buffered saline (PBS) and trypsine–EDTA (both Sigma-Aldrich), MTT (Calbiochem), fluorescein diacetate (FDA) (Invitrogene), propidium iodide (PI) (Fluka) and hydroxyethidine (HE) (Polysciences, USA) were used for cytotoxicity and bioimmunological assays. As a growth media, a Dulbecco's Modified Eagle Medium (DMEM, Invitrogene) containing 5% fetal bovine serum (FBS), was used.

Poly(vinyl alcohol) (PVA, $M_n = 2{,}000$ g/mol) with 75% hydrolyzed vinyl-acetic units was purchased from Sigma-Aldrich. PEI ($M_n = 2{,}000$ g/mol, branched polymer) was purchased as a 50 wt.% aqueous solution from Sigma-Aldrich.

Poly[2-(4-aminophenyl-2-oxazoline)-co-2-ethyl-2-oxazoline] with a molar ratio 10/90 (AEOX10) was prepared previously ($M_n = 10{,}800$, $M_w/M_n = 2.59$) [25, 26].

2.2 Polymerizations

Poly(2-methyl-2-oxazoline) (PMEOX) and PETOX with four different molar masses were prepared by cationic polymerization initiated by methyl *p*-nitrobenzenesulfonate, according to a known procedure [25]. All of the described polymerizations were carried out in 5 mol/l *N*,*N*-dimethylacetamide solution. PETOX containing a pyrene moiety (PETOX-py) was prepared by the bulk polymerization of 2-ethyl-2-oxazoline initiated by 1-(bromoacetyl)pyrene. All of the polymers were terminated by a reaction with a 0.2 M methanolic solution of KOH for 12 h at room temperature.

Polymers used in the cytotoxicity and phagocytosis assays were purified by dialysis on a Spectra/Por Dialysis membrane Nr.6/MWCO 1,000 (Spectrum Laboratories, Inc.) for 24 h. The details of the polymerization conditions are listed in Table 1.

2.3 Cell lines and cultivation

Lymphoid mouse macrophage P388.D1 (Clone 3124) and rat fibroblast-like RAT-2 cell lines (both from ECACC, Europe) were used for the study of the bioimmunological assays or the cytotoxicity, respectively. Cells were collected via trypsinization using trypsine–EDTA (Gibco, BRL) for 1 min at room temperature and were then dispersed in a uniform suspension by adding at least five volumes of growth media containing serum to inactivate the trypsin. Cells were centrifuged at 1,500 rpm for 3 min and then resuspended in fresh growth media (DMEM + 5% FBS for growth of RAT-2 fibroblasts or RPMI with 20% horse serum for growth of P388.D1 cells), and the number of cells was determined using a hematocytometer. Cells were seeded in 12-well culture plate (10^5 cells/well) and incubated in growth medium overnight in a CO_2 incubator at 37°C and $c(CO_2) = 5\%$ before incubation with polymers.

The details of cell culture preparation for phagocytosis, oxidative burst, and determination of cell viability were described previously [26]. For experiments, 5 mg/ml polymer solutions of AEOX10 and PETOX100 were used. Samples for measurements of phagocytosis, oxidative burst and viability staining were taken at four different times of incubation (1, 3, 6 and 24 h).

2.4 Cytotoxicity assay

An MTT cytotoxicity assay was used for estimating the cytotoxic properties of the polymers. Fibroblast cells (10^5 cells per well) were treated with dissolved polymers (1 ml of DMEM growth medium, 0.1–5 mg/ml, in CO_2) in an

Table 1 Polymerization conditions and molecular characteristics of polymers

Polymer	Temperature (°C)	Time (h)	[M]/[I][a]	Yield (%)	M_{theor} (g/mol)	M_n^b (g/mol)	M_w/M_n (g/mol)
PETOX20	80	6	20	100	2000	4300	1.11
PETOX60	80	6	60	100	6700	6700	1.06
PETOX100	80	6	100	100	10000	16200	1.08
PETOX800	80	90	800	85	67300	44700	1.25
PMEOX	80	6	100	100	8500	6100	2.05
PETOX-py	125	4	100	86	10300	13200	1.09

[a] Ratio of starting monomer and initiator concentrations as the theoretical degree of polymerization

[b] Number average molar masses (M_n) were determined by GPC-MALLS using the refractive index increment (dn/dc) equal to 0.117 ml/g

incubator at 37°C and c(CO_2) = 5% for 24 h. MTT (5 mg/ml) were dissolved in PBS (stock solution). The medium was changed, and MTT was added to produce a final concentration of 0.5 mg/ml. The cells were incubated at 37°C for 3 h. The formed purple crystals of insoluble formazane were dissolved in DMSO. The cell viability was determined spectrophotometrically by comparing the absorbance of treated and untreated cells (negative control) at 570 nm. The data were presented as mean ± SEM for triplicates.

2.5 Immunocytometric evaluation of simultaneous phagocytosis and oxidative burst

Phagocytosis, accompanied by a respiratory burst of lymphoid mouse macrophages, was evaluated by flow cytometry (Beckman-Coulter FC 500, CXP software). For each sample, a fluorescence histogram of 10,000 cells was generated and analyzed. Gates were set around the macrophage population to exclude debris. Measurements of phagocytosis, i.e., the ingestion of bacteria, took place under controlled conditions using a fluorescein-labeled opsonized *Saccharomyces aureus* (SPA-FITC) (Molecular Probes, The Netherlands). The metabolic activity was determined via the oxidative burst of the stimulated transformation of originally non-fluorescent HE to fluorescent ethidium, which intercalates DNA to afford red fluorescence (excitation of 488 nm) following SPA-FITC ingestion. Aliquots of macrophages (30 µl) were treated with 5-mg/ml solutions of AEOX10 and PETOX100 and incubated with HE (15.75 mg in 5 ml of DMF) for 15 min at 37°C. Following treatment with SPA-FITC for 15 min at 37°C, the reaction was stopped with ice. A subsequent lysis was performed for 15 min with an ice-cold ammonium chloride/potassium chloride (ACK) lysis buffer (200 ml deionized water; 1.658 g NH_4Cl; 0.2 g $KHCO_3$; 7.4 mg Na_2EDTA; pH 7.2–7.4). The mean % of phagocytic cells represents the percentage of granulocytes ingesting at least one SPA-FITC particle, the mean % of respiratory burst represents the percentage of granulocytes tagged by ethidium and the mean metabolic activity % represents the percentage of granulocytes ingested at least one SPA-FITC and were tagged by ethidium.

2.6 Viability assay

Macrophage viability following treatment with 5 mg/ml solutions containing AEOX10 and PETOX100 was evaluated via PI/FDA staining. Briefly, 50 µl of freshly distilled water with diluted FDA (5 mg/ml stock solution in acetone, stored at −20°C; before use, 20 µl of this solution was diluted with 4.2 ml of distilled water) and 50 µl of PI (0.02 mg/ml in distilled water) were added to a 100-µl cell suspension. After 20 min of staining, the macrophage suspension was washed with saline. The fluorescence of FDA^+ (FDA-stained, viable cells) and PI^+ cells (PI penetrates cell membranes of dying or dead cells) was evaluated by flow cytometry (FL1 vs. FL3) using a Beckman-Coulter FC 500 flow cytometer (Beckman Coulter Inc., Fullerton, California, USA).

2.7 Microscopy

A confocal laser scanning microscope LSM 700 (Zeiss, Germany) was used with 20×/0.75 Plan-Apochromat objective, 405 nm laser line for excitation.

The proliferation and morphologies of RAT-2 fibroblasts-like cells were analyzed by optical microscopy (XDS-IM1, Optika microscopes) using a digital camera (Canon, DS 126071) with 10× and 2.5× objectives.

2.8 Analytical measurements and methods

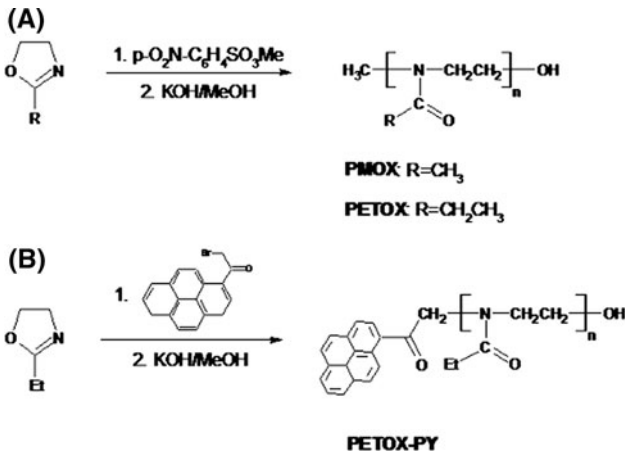
The ^1H and ^{13}C NMR spectra of all of the compounds were recorded at room temperature using a Varian VXR-400 in DMSO-d_6 solutions. UV spectra were analyzed with an UV/Vis spectrophotometer (Shimadzu 1650 PC), and fluorescence measurements were recorded on a spectrofluorophotometer (Shimazu RF-5301). The emission spectra of treated cells were measured using concentrations of 10^{-7} mol/l in PBS with a slit of 3 × 3 nm and excitation wavelength of 340 nm. The emission spectra of pyrene-labelled polymer (PETOX-py) were conducted in PBS and in chloroform using concentrations of 10^{-7} mol/l.

The molar masses and dispersities of the obtained products were determined by GPC-MALLS—gel permeation chromatography with a differential refractive index detector Dn-2010, obtained from WGE Dr. Bures, and a multiangle laser-light scattering detector DAWN EOS, purchased from Wyatt Technologies Measurements were performed using Polymer Standard Service (PSS) columns: SDV 1×10^5 Å + 1×10^3 Å + 2×10^2 Å, in THF, at 35°C and with a nominal flow rate of 1.0 ml/min. The results were evaluated by ASTRA 4.73 (Wyatt Technology) and WINGPC Unity from PSS software.

3 Results

3.1 Polymer synthesis and characterization

Poly(2-oxazolines) were prepared by the living cationic polymerization using two different initiators. In the first case, the polymerizations were initiated by methyl *p*-nitrobenzenesulfonate, and *N*,*N*-dimethylacetamide was used as the solvent (Scheme 1a). For the preparation of



Scheme 1 Cationic polymerization of 2-alkyl-2-oxazolines initiated by methyl *p*-nitrobenzenesulfonate (**a**) and 1-(bromoacetyl)pyrene (**b**)

fluorescent probe-containing PETOX, 1-(bromoacetyl)pyrene was used as the initiator (Scheme 1b). The molar masses and dispersities were determined by GPC-MALLS. For molar mass determination of all PETOX samples and PMEOX sample the refractive index increment (dn/dc) equal 0.117 ml/g was used. This could result in some discrepancy of theoretical and experimental values of molar masses for PMEOX and PETOX-py samples. Nevertheless, as shown in Table 1, most of molar masses are in the good agreement with the theoretical values, and the dispersity indexes of all polymers were in the range 1.05–1.30. This data indicated the living character of polymerizations. The only exception to this characteristic was with PMEOX; the dispersity was equal to 2.05. The structures of all of the polymers were confirmed by NMR spectroscopy analyses (Figs. 1, 2, 3 in Supplementary Data).

Aromatic 2-oxazolines provide poly(2-oxazolines) that are insoluble in water. Therefore, we used their copolymers with 2-ethyl-2-oxazolines. The syntheses of these copolymers, containing different amounts of 4-aminobenzoyl moieties (Scheme 2), have been described previously [26]. It was found that copolymers containing less than 20 mol% of the 4-aminobenzoyl moiety are soluble in water. Thus, the copolymer AEOX10, which contained 10 mol% of those aromatic building units, was chosen for the use in cytotoxicity and immunoefficiency studies.

3.2 Cytotoxicity assays

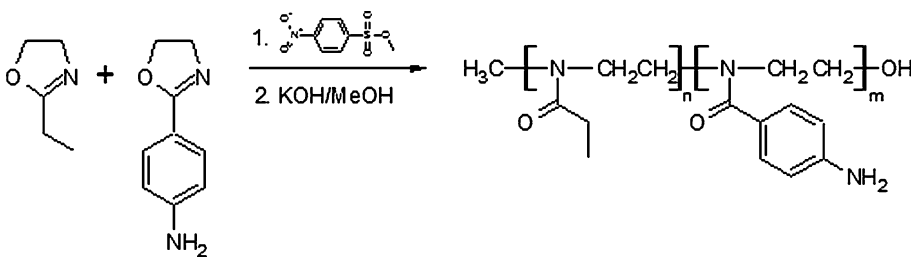
An MTT assay (Fig. 3 in Supplementary Data) was conducted to estimate the cytotoxicity of all tested poly(2-alkyl-2-oxazolines). This method represents a standard assay of cell viability, which is based on the colorimetric analyses of living cells. Three concentrations (0.5, 1 and 5 mg/ml) of the studied polymers were tested for their influence on cell proliferation and viability. Non-treated cells were used as a control. As demonstrated in Fig. 1, both polymers did not influence the cell viability. It could be concluded that PMEOX and PETOX100, which differ in chain length, are non-cytotoxic to cells after 24 h of incubation also in high concentrations.

Pyrene-labelled PETOX (PETOX-py) was employed for the visualization of polymer within the cell. Therefore, the cytotoxicity of PETOX-py was also compared to PETOX100 and PMEOX, and PETOX-py was observed to be non-cytotoxic after 24 h (Fig. 1).

To confirm the cytotoxicity of PMEOX and PETOX100, a 48-h incubation period was administered. The results were compared with two other polymers the PVA and branched PEI; PVA represents a biocompatible polymer, and PEI is a cytotoxic polymer. The cytotoxicity of the monomer was also measured. Figure 2 shows that the longer incubation times did not influence cell viability if incubated with low (0.5 mg/ml) or high (5 mg/ml) concentrations of PMEOX or PETOX100. As expected, the biocompatible polymer, PVA, did not affect cell viability in high concentrations. The cytotoxicity of PEI and the monomer is also evident in low concentrations and increases with incubation time and concentration. These results showed low cytotoxicity of PETOX100 and PMEOX even after longer incubation times at low or high concentrations compared to positive (PVA) and negative (PEI) controls.

Furthermore, we compared the cytotoxicity of polymers containing aliphatic substituents (PETOX100 and PMEOX) with the selected copolymer containing aromatic segments (AEOX10). The cell viability of AEOX10 was determined previously [26] (Fig. 4 in Supplementary Data). The measurements indicated that the presence of a benzene ring has no affect on the growth and proliferation of cells and that the values of cytotoxicity measured in the

Scheme 2 Copolymerization of 2-ethyl-2-oxazoline with 2-(4-aminophenyl-2-oxazoline)



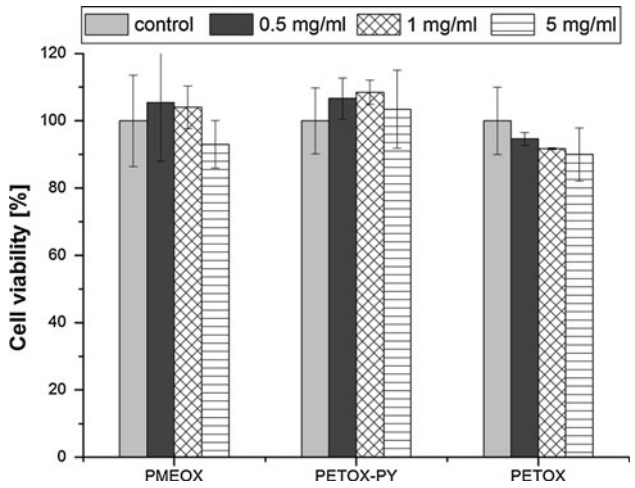


Fig. 1 Cell viability determined from the MTT analyses of cells treated with varying concentrations of poly(2-methyl-2-oxazoline) (PMEOX), poly(2-ethyl-2-oxazoline) (PETOX100) and poly(2-ethyl-2-oxazoline) containing a pyrene moiety (PETOX-py). The data represent the means ± standard error of the mean (SEM) of triplicate values

MTT test were on the same level as in the case of PETOX100 and PMEOX [26].

The morphology of the treated cells was monitored by the mean of optical microscopy. In all cases, cells showed normal morphologies during the incubation time (Fig. 5 in Supplementary Data).

The PETOX100 used for previous experiments had a molar mass of 1.6×10^4 g/mol. To confirm the influence of the oxazoline unit on cell viability, polymers with lower and higher molar masses were prepared and tested (Table 1). Cytotoxicity was examined using the MTT assay, as previously described. As shown in Fig. 3, polymers with molar masses lower than 1.6×10^4 g/mol exhibit slightly higher cytotoxicity, whereas polymers with higher molar masses did not affect the viability of cells.

3.3 Fluorescence microscopy and steady-state fluorescence

For these purposes, a pyrene probe was used to label the polymers within the cells during cytotoxicity analyses. Pyrene-based probes are in a class of hydrophobic dyes and can be used not only for the visualization and analysis of polymer concentrations within cells but also for the investigation of the environment polarity and the formation of agglomerates. Fluorescence of PETOX-py in PBS and in chloroform is shown on Fig. 4. Fibroblasts were treated with labeled polymer similarly to the MTT cytotoxicity assays. Cells treated with different concentrations of labeled polymer were harvested, and the fluorescence was measured by spectrofluorimeter (Fig. 5). In parallel, the treated cells were washed and investigated by laser scan

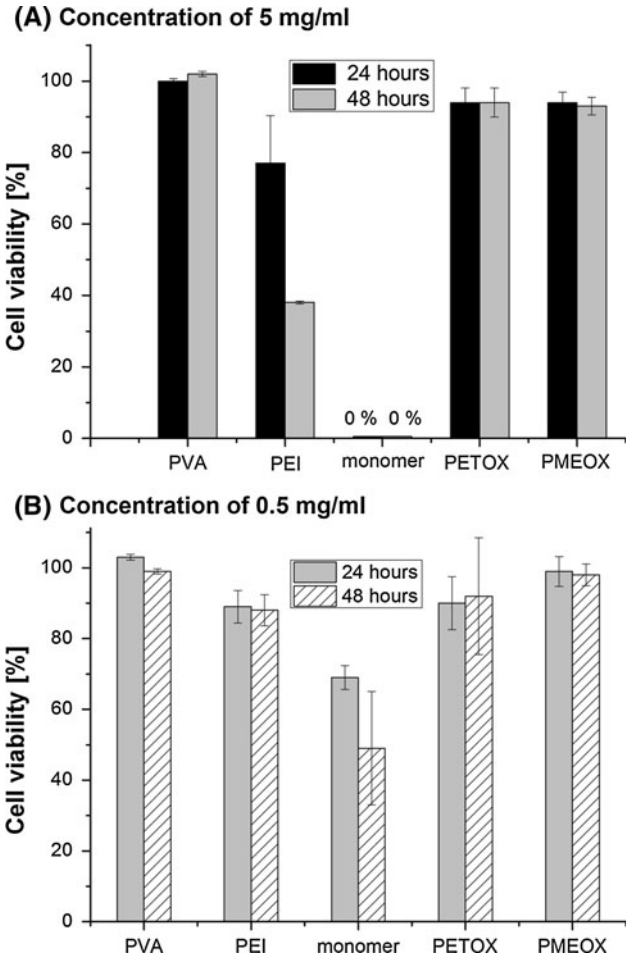


Fig. 2 A comparison of cell viability with 2-oxazoline polymers, the monomer 2-ethyl-2-oxazoline and positive PVA and negative PEI controls in two concentrations: **a** concentration of 5 mg/ml, **b** concentration of 0.5 mg/ml. The data represent the means ± standard error of the mean (SEM) of triplicate values

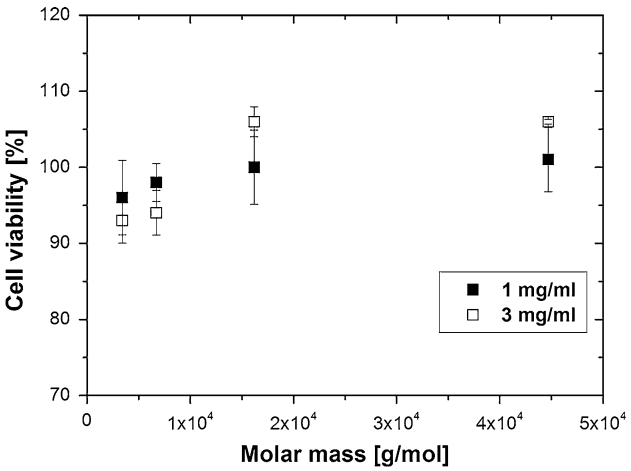


Fig. 3 The dependence of cell viability with PETOX on the molar mass (1 and 3 mg/ml). The data represent the means ± standard error of the mean (SEM) of triplicate values

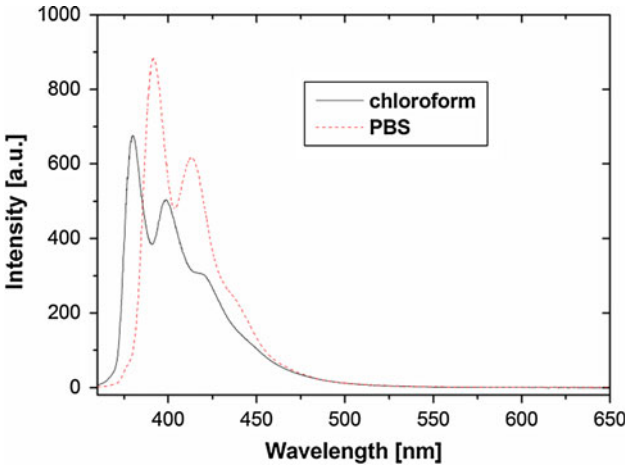


Fig. 4 Fluorescence spectra of 2-ethyl-2-oxazoline labeled with a pyrene probe (PETOX-py) in PBS medium and chloroform ($c = 10^{-7}$ mol/l)

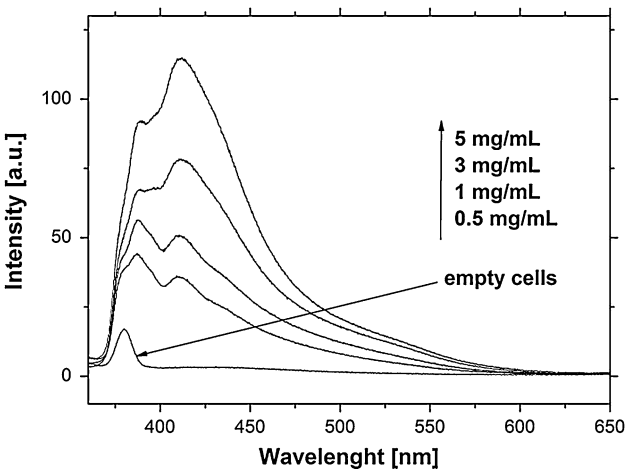


Fig. 5 Fluorescence spectra of the cells treated with PETOX in different concentrations measured in PBS

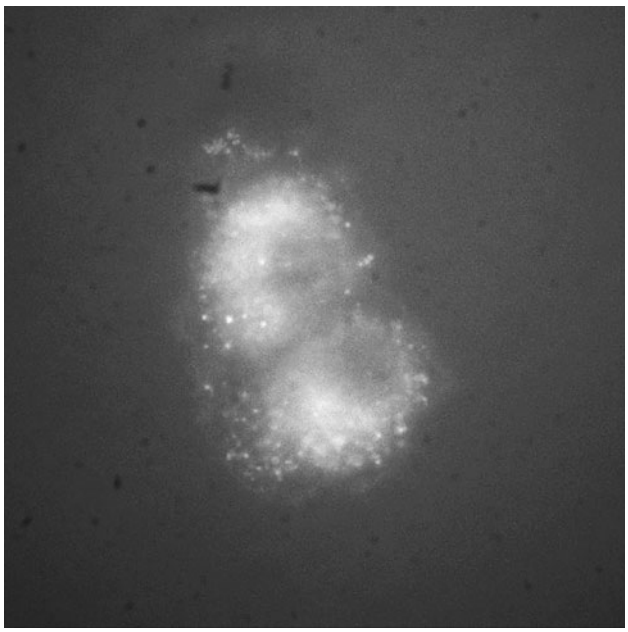


Fig. 6 A micrograph of the cells treated with PETOX-py

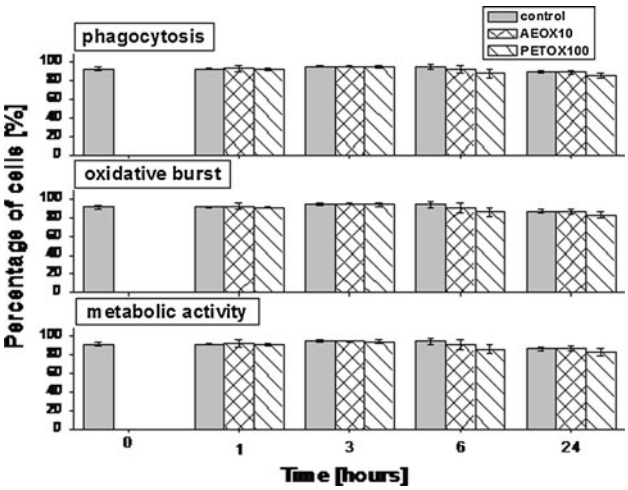


Fig. 7 The influence of AEOX10 and PETOX100 (5 mg/ml) on phagocytic effectiveness, oxidative burst and metabolic activity (phagocytosis and oxidative burst) of macrophages. The data represent the means ± standard error of the mean (SEM) of duplicate values

microscopy (Fig. 6). Both methods confirmed the accumulation of polymer within the cells. The concentration of polymer within the cells was dependent on the concentration of polymer in the environment, as shown by spectrofluorimeter analyses.

3.4 Immunological assays

To examine the effects of AEOX10 and PETOX100 at high concentrations (5 mg/ml) on macrophage functionality, mouse macrophages were assayed for induced changes of phagocytic capability, effectiveness of respiratory burst (Fig. 7) and viability using flow-cytometric quantitation (Fig. 8). As a consequence of AEOX10 and PETOX100 interactions with macrophages, the slight increase of phagocytosis after 3 h was observed; this peak activity was also demonstrated with untreated cells, suggesting natural cell behavior. When compared both substances with baseline values, this increase was comparable: 2.98% with AEOX10 and 2.16% with PETOX100, the increase of the untreated population was 3.22%. The kinetics of phagocytosis at 6 and 24 h compared with the baseline values revealed decreasing tendency, more profound with PETOX100 (7.6% decrease) in the comparison with AEOX10—only 3.53% decrease, the last one is comparable with spontaneous decline of untreated cells (3.95%

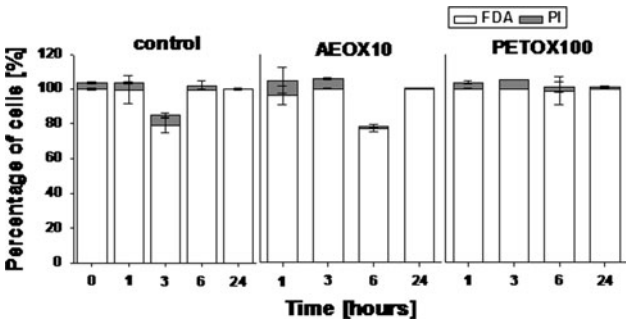


Fig. 8 Timed kinetics of macrophage cell viability under AEOX10 and PETOX100 treatment. The data represent the means ± standard error of the mean (SEM) of duplicate values

decrease). The metabolic activity and oxidative burst measurements are in the agreement with observed time-kinetics of phagocytosis of AEOX10 and PETOX100 during interactions with macrophages. To evaluate potential impairment of cell metabolism by AEOX10 and PETOX100 treatment, quantitative tests on cell viability were performed (Fig. 8). Only slight decrease of FDA activity was observed after 6 h treatment with AEOX10 and PETOX100, with subsequent spontaneous improvement demonstrable at 24 h (100% of FDA activity). The same kinetic trend could be seen also with untreated control. The maximum 8.6% of PI^+ cells were observed after the first hour following AEOX10 administration. The absolute minimum of PI^+ cells was measured 24 h after AEOX10 and PETOX100 exposition (0.5 and 0.4%, respectively).

4 Discussion

All of the polymers were prepared by the cationic polymerization of 2-alkyl-2-oxazoline compounds, which are classified as living polymerization processes. This method is employed to control the molar masses of the resultant polymers. The prepared polymers, PMEOX, PETOX100 and PETOX-py were tested for cytotoxicity using MTT assay. All polymers were biocompatible for RAT-2 cells after 24 h of the incubation also in high polymer concentrations. However, compared to PETOX100, the cell proliferation appeared to be less affected by PMEOX and PETOX-py (Fig. 1). This result could be due to the difference in molar mass between PMEOX and PETOX. However, differences are not statistically significant in the comparison with standard deviations of measurements. The increase of cytotoxicity with decreasing molar mass can be explained by the cytotoxic activity of the 2-oxazoline unit. Shiori et al. [27] discovered the relationship between the cytotoxicity of cyclic peptides of marine origin and the oxazoline unit content. This result was supported by the observance of the very low viability of cells treated with 2-ethyl-2-oxazoline as monomer (Figs. 2a, b) and by polymers with lower molar masses than PETOX100 (Fig. 3).

The results were compared with two other polymers the PVA and PEI; PVA represents a biocompatible polymer, and PEI is a cytotoxic polymer. The cytotoxicity of the 2-ethyl-2-oxazoline as one type of monomers was also measured. Figure 2 shows that the longer incubation times did not influence cell viability if incubated with low (0.5 mg/ml) or high (5 mg/ml) concentrations of PMEOX or PETOX100. Notably, monomer traces can rapidly increase the cytotoxicity of the polymer, and therefore, thorough purification is necessary before testing its cytotoxicity.

The toxicity of compounds containing benzene ring was shown for different aromatic drugs [28, 29]. In spite of these results, our measurements indicated that the presence of a benzene ring in AEOX10 [26] has no affect on the growth and proliferation of cells and that the values of cytotoxicity measured in the MTT test were on the same level as in the case of PETOX100 and PMEOX.

Fluorescence spectroscopy serves as a very convenient method for the visualization and quantification of accumulation (uptake) of polymers in cells. Recently, a confocal laser-scanning microscope was used for the study of PETOX uptake into fibroblasts. For visualization, fluorescein-labelled polymers were used [21]. They prepared fluorescein-labelled PETOX using fluorescein as the termination step. We used the analogous approach for the visualization and quantification of the presence of 2-oxazoline-based polymers after treatment with fibroblast cells. Fluorescent microscopy and CLSM were used as independent visualization methods and both confirmed the accumulation of polymer within the cells. The concentration of polymer within the cells was dependent on the concentration of polymer in the environment, as shown by spectrofluorimeter analyses. Furthermore, the laser scanning showed that polymer was not located within whole cells but either formed aggregates or could be accumulated within a specific organelles. This is a topic for further investigation.

The capability of macrophages to phagocyte the foreign substances, particles, microbes etc. with subsequent induction of respiratory burst represents the hallmark of their intact cell-metabolism [5, 6]. The macrophages challenged by AEOX10 and PETOX100 did not exert decreased functionality; the phagocytic and metabolic activities were comparable with that of untreated (control) cells. Moreover, neither polymer significantly affected the oxidative burst (Fig. 7) what is unique property of activated phagocytes with capacity to generate and release substantial amounts of reactive oxygen species. Evidently, a dosage of 5 mg/ml of both substances represents a safe dose and did not provide interference with the functional properties of the phagocytic

cells. Additionally, as evidenced by cell viability assays, practically no cell injury following AEOX10 and PETOX100 treatment had been observed. Almost 100% macrophage FDA activity, characteristic for cells without any cell-impairment was revealed 24 h after AEOX10 and PETOX100 treatment (Fig. 8).

5 Conclusion

Polymers prepared by the cationic polymerizations of 2-alkyl- and 2-aryl-2-oxazolines were used for treatment of two model cell lines (macrophages and fibroblasts) and responses were monitored using different methods. Two independent methods were used to assess the cytotoxicity of different oxazoline-based polymers: (i) an MTT study using RAT-2 fibroblasts and (ii) FDA–PI viability assays using P388.D1 macrophages. These tests indicated a high level of cell viability and confirmed a low affect of the presence of polymers on cell growth and proliferation. Additionally, fluorescence spectroscopy and microscopy confirmed the accumulation of polymers in the cells. The mechanism of polymer metabolism will be the subject of further study.

The influence of these polymers on the immunological activity of macrophages was also studied. Overall, AEOX10 and PETOX100 exhibited no immunosuppressive influence or undesirable effects on the macrophage activity. The investigation of macrophage viability indicated that AEOX10 and PETOX100 are safe and biocompatible. On the basis of the cytotoxicity and immunological assays, we can conclude that AEOX10 and PETOX100, even at high concentrations of 5 mg/ml, do not exert any immunosuppressive effect or toxic behavior in cells. Assessment of macrophage activity in the presence of a tested polymer extends the methods for the evaluation of polymer biocompatibility. Such measurements are necessary especially for using the polymer in further immunological applications, e.g. in the construction of vaccines. The influence of oxazoline-based polymers on cellular immunity, specifically on macrophage cytokine release, will be published later.

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