A straightforward route to superhydrophilic poly(2-oxazoline)s via acylation of well-defined polyethylenimine

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Abstract

Herein, we describe a new method for the synthesis of superhydrophilic poly(2-alkyl-2-oxazoline)s (PAOx) from poly(2-ethyl-2-oxazoline) (PEtOx). A well-defined linear polyethylenimine was prepared from PEtOx by controlled acidic hydrolysis of its side-chains followed by re-acylation with different carboxylic acids. Using this protocol, we obtained a series of new hydrophilic PAOx containing side-chain ether groups with potential in the biomaterials science. The relative hydrophilicity of polymers was assessed, revealing that poly(2-methoxymethyl-2-oxazoline) (PMeOMeOx) is the most hydrophilic PAOx to date. Additionally, the amorphous poly(2-methoxy-ethoxy-ethoxymethyl-2-oxazoline) (PDEGOx) shows the lowest reported glass transition temperature (-25 °C) within the PAOx family to date. The biomedical potential of prepared polymers was further fortified by an *in vitro* cytotoxicity study, where all polymers appeared to be non-cytotoxic. The described synthetic protocol is universal and can be extremely versatile, especially for PAOx that are difficult to prepare by conventional cationic ring opening polymerization due to the monomer interference and/or degradation.

Introduction

The synthesis of well-defined biopolymers continues to attract substantial attention in chemical and biomedical research, playing the key role in the construction of systems for drug/gene delivery or tissue engineering.[1-4] In the last decade, poly(2-alkyl-2-oxazoline)s (PAOx) gained considerable popularity due to their synthetic versatility and tunable properties.[5-11] The polymer properties can be modulated by selecting appropriate side-chain substituents, chain-end functional groups and the polymer chain length.[12] Within this class, poly(2-ethyl-2-oxazoline) (PEtOx) and poly (2-methyl-2-oxazoline) (PMeOx) have found widespread biomedical applications resulting from their hydrophilicity, biocompatibility,[6,13-15] non-immunogenicity[16] and flexibility.[17] These properties are often superior to those of the other polymers extensively used in biomedical research (e.g., polyethylene oxide or poly(*N*-(2-hydroxypropyl) methacrylamide)).[16,18]

Well-defined PAOx can be synthesized by living cationic ring-opening polymerization (CROP) of their respective monomers with alkyl tosylates being the most common initiators.[12] The polymerization is terminated by nucleophiles providing an easy route for introduction of chain-end functionality by simply selecting the appropriate terminating agent. Under strictly inert conditions, the CROP of 2-oxazolines proceeds without termination and chain transfers, yielding polymers with narrow molar mass distribution. The polymerization time can be substantially reduced by employing pressurized high temperature conditions, e.g. using microwave irradiation, yielding polymers within a couple of minutes.[19-20] Finally, the potential of PAOx was further enhanced by the recent discovery of a synthetic procedure for the preparation of defined high molar mass PAOx.[21]

Despite the synthetic versatility and broad applicability, the synthesis of PAOx still presents some major challenges. As the propagating oxazolinium chain-ends react with nucleophiles, monomers bearing such groups (e.g., free amines, alcohols, thiols, carboxylic acids) cannot be polymerized by CROP. This can be solved by employing suitable protective groups, followed by the post-polymerization deprotection.[22-23] Furthermore, CROP cannot be utilized for monomers that interfere with standard polymerization process (e.g., monomers bearing aliphatic bromide or tosylate) or monomers rapidly degrading at elevated temperature. As the preparation of new, highly functionalized PAOx is desirable, the search for alternative synthetic strategies provides an appealing quest in polymer chemistry.

The living character of CROP allows us to synthesize a wide range of copolymer architectures including statistical, gradient or block copolymers.[24-25] Recently, Mees et al reported an alternative route to PAOx copolymers consisting in the partial acidic hydrolysis of PEtOx homopolymer to statistical poly(2-ethyl-2-oxazoline)-*co*-polyethylenimine (PEtOx-PEI) copolymers, that were further modified by acylation using methyl succinyl chloride.[26-27] This post-polymerization strategy might be the only effective way to synthesize statistical copolymers, where different reactivities of respective monomers lead to copolymers with strong gradient of composition (e.g., 2-phenyl-2-oxazoline with MeOx).[25] In another study, the PEtOx-PEI copolymer was exploited in the synthesis of glycopolymers using reductive amination reaction.[28]

Despite many reports on full hydrolysis of PAOx yielding well-defined linear polyethylenimine (PEI),[29-31] the reverse reaction, i.e., the complete acylation of PEI yielding defined PAOx homopolymers was not yet employed for the preparation of novel PAOx. The reacylation of PEI obtained from hydrolysis of poly(2-methyl-2-oxazoline) with acetic anhydride was, however, already shown in one of the first papers reporting the CROP of 2-oxazolines.[32] This reacetylation method of PEI towards PAOx synthesis might be an elegant alternative to the conventional cationic ring-opening polymerization, especially in the case of unstable monomers or functional monomers interfering with the polymerization process. Herein, we describe such a protocol for the straightforward synthesis of defined functional PAOx, via acylation of PEI, that can not be straightforwardly synthesized *via* the monomer. To demonstrate the versatility of this reacetylation route, a series of new hydrophilic PAOx was prepared and their physical, chemical and biological properties were studied by different techniques with emphasis on their potential in biomedical research. More specifically, a series of PAOx having defined oligoether side chains is reported, which was inspired by the high hydrophilicity and good antifouling behavior of the structurally related methoxyethyl-substituted polypeptoids.[33-34] Furthermore, our attempts to prepare the 2-methoxyethyl-2-oxazoline monomer revealed that it is unstable and undergoes spontaneous elimination of methanol, partially, yielding 2-vinyl-2-oxazoline (see Supporting Information), indicating the need for an alternative pathway to prepare such PAOx with oligoether side chains.

EXPERIMENTAL SECTION

Materials.

2-Ethyl-2-oxazoline (EtOx) was kindly donated by Polymer Chemistry Innovation and was distilled over CaH_2 before use. Methyl *p*-toluenesulfonate (MeOTs) was obtained from Sigma-Aldrich and was distilled from CaH_2 prior to use. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Sigma-Aldrich and was used as received. Acetonitrile (Sigma-Aldrich) was purified over aluminum oxide using a solvent purification system from J.C. Meyer. All other chemicals, including acetic acid, 2-ethanolamine, 3-methoxypropionitrile, methoxyacetic acid, ethoxyacetic acid, 3-methoxypropionic acid, 3-ethoxypropionic acid, [2-(2-methoxyethoxy)ethoxy]acetic acid, *N*,*N*-diisopropylethylamine (DIPEA), fluorescein-5-isothiocyanate (FITC) and DL-dithiothreitol (DTT) were purchased from TCI Europe and were used as received. The PEtOx DP 200 and 400 were synthesized according to literature procedures.[35]

Synthesis of 2-methoxyethyl-2-oxazoline (MeOEtOx)

A suspension of $Zn(OAc)_2.2H_2O$ (10.97 g, 0.05 mol, 0.05 equiv.) in 2-ethanolamine (80.7 g, 1.32 mol, 1.2 equiv.) and 3-methoxypropionitrile (93.7 g, 100 ml, 1.10 mol, 1 equiv.) was heated to 130°C for 14 hours until no 3-methoxypropionitrile was detected anymore by gas chromatography (GC). The obtained product consisted of a 1:2 mixture of MeOEtOx and 2-vinyl-2-oxazoline (VinOx) resulting from elimination of methanol as determined by ^{1}H-NMR spectroscopy (Figure S3). Attempts to purify the MeOEtOx by distillation led to further elimination of methanol. Therefore, this route was not further pursued.

Synthesis of poly(2-ethyl-2-oxazoline) (PEtOx)

In the glove-box, the monomer EtOx (10 g, 101 mmol) and the initiator MeOTs (152 µL, 1.0 mmol, [EtOx]:[MeOTs] = 100:1) were dissolved in dry acetonitrile (15.2 mL) and stirred at 80 °C for 3 h. Then, a sample for GC was taken and the mixture was mixture was cooled down to room temperature, followed by termination with solid NaN_3 (328 mg, 5.1 mmol) overnight. The GC analysis revealed 79 % conversion of the monomer. The reaction mixture was precipitated in cold diethyl ether, filtered and dried under reduced pressure. The crude polymer was dissolved and purified by dialysis (MWCO = 1 kDa) against distilled water followed by freeze-drying to obtain PEtOx (7.3 g, 73 %) as a white powder.

Synthesis of poly(ethylene imine) (PEI)

PEtOx (8 g) was dissolved in aqueous hydrochloric acid (~18 wt.%, 80 mL) and refluxed overnight (14 h) under argon atmosphere. All volatiles were then removed under vacuum and the crude PEI hydrochloride was suspended in ice-cold distilled water (80 mL). Ice-cold aqueous sodium hydroxide (2 M) was added dropwise to the suspension until it dissolved, but with further addition of NaOH the free base of PEI precipitated at pH 10-11. The precipitate was filtered, washed with distilled water, recrystallized twice from the same solvent and dried under high vacuum to obtain PEI as a white powder (3.1 g, 88 %). M_n(SEC) = 3.0 kDa, $Đ$(SEC) = 1.09.

Acylation of PEI

General procedure A. PyBop (4.84 g, 9.3 mmol) and the corresponding carboxylic acid (**Table 1**, 9.3 mmol) were dissolved in dry *N*,*N*-dimethyl formamide (DMF, 30 mL). DIPEA (2.43 mL, 14.0 mmol) was added dropwise and the mixture was stirred at room temperature. After 2 min, the mixture was transferred into a solution of PEI (200 mg, 4.7 mmol amine groups) in dry DMF (30 mL), followed by stirring at room temperature overnight (14 h) under argon atmosphere. The solvent was evaporated under reduced pressure and the polymer was isolated by dialysis (MWCO = 1 kDa) against distilled water followed by freeze-drying. The full conversion of amines was confirmed by ^{1}H-NMR spectroscopy and the Kaiser test. In the latter method, the prepared polymers were dissolved in 1 M solution of ninhydrin in ethanol (c_{pol} = 10 mg mL^{-1}) and incubated at 50 °C for 24 h. The absence of coloration indicated full conversion of the amino groups.

Poly(2-methyl-2-oxazoline) (PMeOx) was synthesized according to the general procedure using acetic acid in 78 % yield. ^{1}H NMR (ŭ ĭ$_2$O, 400 MHz, ppm): 3.63 ï 3.45 (m, 4H), 2.20 ï 1.94 (s, 3H)

Poly(2-methoxymethyl-2-oxazoline) (PMeOMeOx) was synthesized according to the general procedure using acetic acid in 86 % yield. ^{1}H NMR (ŭ ĭ$_2$O, 400 MHz, ppm): 4.27 ï 4.09 (m, 2H), 3.63 ï 3.46 (m, 4H), 3.41 (s, 3H).

Poly(2-ethoxymethyl-2-oxazoline) (PEtOMeOx) was synthesized according to the general procedure using acetic acid in 79 % yield. ^{1}H NMR (ŭ ĭ$_2$O, 400 MHz, ppm): 4.36 ï 4.18 (m, 2H), 3.70 ï 3.45 (m, 6H), 1.28 ï 1.23 (m, 3H).

Poly(2-methoxyethyl-2-oxazoline) (PMeOEtOx) was synthesized according to the general procedure using acetic acid in 90 % yield. ^{1}H NMR (ŭ i n, 400 MHz, ppm): 3.73 ï 3.60 (m, 2H), 3.58 ï 3.35 (m, 4H), 3.29 (s, 3H), 2.68 ï 2.40 (m, 2H).

Poly(2-ethoxyethyl-2-oxazoline) (PEtOEtOx) was synthesized according to the general procedure using acetic acid in 85 % yield. ^{1}H NMR (ŭ in $CDCl_3$, 400 MHz, ppm): 3.78 ï 3.43 (m, 8H), 2.74 ï 2.55 (m, 2H), 1.21 ï 1.09 (m, 3H).

Poly(2-[methoxy-ethoxy-ethoxymethyl]-2-oxazoline) (PDEGOx) was synthesized according to the general procedure using acetic acid in 71 % yield. ^{1}H NMR (ŭ in $CDCl_3$, 400 MHz, ppm): 4.35 ï 4.25 (m, 2H), 3.74 ï 3.47 (m, 12H), 3.36 (s, 3H).

Labeling of polymers with fluorescein

Azide-functionalized PAOx (80 mg) and DTT (10 equiv of azide) were dissolved in phosphate buffered-saline (PBS, 2 mL, pH = 7.4, c = 150 mM) and stirred at room temperature overnight (16 h). The resulting amine-functionalized PAOx was recovered by gel filtration on a Sephadex PD-10 column using distilled water as an eluent and isolated by freeze-drying. The obtained solid polymer (72 - 81 mg) and FITC (2 equiv) were dissolved in DMF (0.5 mL) followed by addition of triethylamine (3 equiv). After stirring at room temperature overnight, the reaction mixture was diluted with distilled water (0.5 mL) and separated by gel filtration on a Sephadex PD-10 column using distilled water as an eluent and isolated by freeze-drying the polymer fractions. This separation procedure was repeated to obtain the pure fluorescein-labeled PAOx samples (64 ï 77 mg) as dark-orange solids.

Characterization of polymers.

Gas chromatography (GC) was used to monitor the CROP of EtOx employing an Agilent 7890A system equipped with a VWR Carrier-160 hydrogen generator and an Agilent HP-5 column of 30 m length and 0.32 mm diameter. An FID detector was used, and the inlet was set to 240 °C with a split injection ratio 25:1. Hydrogen was used as carrier gas at a flow rate of 2 mL min^{-1}.

Size exclusion chromatography (SEC) was used to determine the molecular weights (M_m - mass-averaged molecular weight, M_n - number-averaged molecular weight) and dispersity ($Đ = M_m/M_n$) of the prepared polymers. This was performed using an HPLC Ultimate 3000 system (Dionex, USA) equipped with a SEC column (TSKgel SuperAW3000 150 × 6 mm, 4 ε mfor PAOx, respectively TSKgel G5000PWXL-CP 300 × 7.8 mm, 10 ε mfor PEI). Three

detectors, UV/VIS, refractive index (RI) Optilab®-rEX and multi-angle light scattering (MALS) DAWN EOS (Wyatt Technology Co., USA) were employed; with a methanol and sodium acetate buffer (0.3 M, pH 6.5) mixture (80:20 vol%, flow rate of 0.5 mL min^{-1}) as mobile phase. A differential refractometer (Wyatt Optilab T-rEX) was used to determine the dn/dc values of the polymers (**Table S1**). The molecular weights of the fluorescein-labeled PAOx were determined by SEC using an Agilent 1260-series HPLC system equipped with a 1260 ISO-pump, a 1260 automatic liquid sampler, a thermostatted column compartment at 50 ÁC equipped with 2 columns and a pre-column in series, a 1260 diode array detector and a 1260 RI detector. The used eluent was DMA containing 50 mM of LiCl at a flow rate of 0.5 ml min^{-1}. Molar mass values and *Đ* values are calculated against narrow dispersity PMMA standards.

High performance liquid chromatography (HPLC) analyses were performed with a HPLC Ultimate 3000 system (Dionex, USA) using a reverse-phase column (Chromolith Performance RP-18e 100 × 4.6 mm, Merck, Germany) and multi-angle light scattering (MALS) DAWN EOS detection. A gradient of acetonitrile/water from 5 % to 95 % in 10 min was used as a mobile phase (flow rate of 2 mL min^{-1})

Nuclear magnetic resonance (NMR) spectra were measured with a Bruker Advance MSL 400 MHz NMR spectrometer. All chemical shifts are given in ppm.

Fourier transformed infrared (FTIR) spectra were recorded on an IRAffinity-1 Shimadzu FT-IR spectrophotometer with MIRacle Attenuated Total Reflectance Attachment at resolution of 4 cm^{-1} accumulating 50 scans.

The cloud point temperature (T_{cp}) of PEtOEtOx was measured on a Crystal 16™ parallel crystallizer turbidimeter (Avantium Technologies) connected to a recirculation chiller at concentration c_{pol} = 10 mg mL^{-1} and heating/cooling rate of 0.5 °C min^{-1}. The T_{cp} was reported as the temperature with 50 % transmittance in the heating run. Additionally, the T_{cp} was measured by dynamic light scattering (DLS) using a Zetasizer NanoZS instrument, Model ZEN3600 (Malvern Instruments, UK). The polymer was dissolved in distilled water (c_{pol} = 10 mg mL^{-1}) and filtered through an 0.22 µm PVDF syringe filter. The total light scattering intensity was determined at a scattering angle of *d*= 173° and the DTS (Nano) program was used to evaluate the data. After each increase in temperature (0.5 °C step), the sample was equilibrated for 5 min followed by the DLS measurement. The T_{CP} corresponds to the onset of the increase of the scattered light intensity.

Differential scanning calorimetry (DSC) was performed on a Mettler-Toledo DSC1 module in a nitrogen atmosphere with a heating/cooling rate of 5 °C min^{-1}. Indium was used as a standard for temperature and enthalpy calibrations. The values of the glass transition temperature (T_g) were determined from the second heating run.

Thermogravimetric analyses (TGA) were performed on a Mettler-Toledo TGA/SDTA851e in a nitrogen atmosphere in the range from 25 °C to 800 °C with a heating rate of 10 °C min^{-1}. The samples were dried in a vacuum oven at 40 °C for 24 h prior to use.

The fluorescein content in PAOx-fluorescein conjugates was measured by UV/VIS spectrometry (Evolution 220 Spectrometer, Thermo Scientific) in sodium carbonate buffer (pH = 9.2, c = 0.15 M) at 25 °C (ε = 11 500 l mol^{-1} cm^{-1}; λ = 488 nm). All measurements were performed in triplicate.

Partition coefficient of PAOx-fluorescein conjugates was determined by extraction experiment according to a literature procedure.[36] Briefly, PAOx-fluorescein conjugates (2 mg) were dissolved in PBS (pH = 7.4, 150 mM) to achieve equimolar solutions with comparable absorbance at 488 nm (A_0). From this stock solutions, 2 mL were aliquoted into the clean vial followed by addition of either 1-octanol or dichloromethane (2 mL). The sealed vial was mixed by shaking for at least 2 h followed by standing for another 2 h to allow the phase separation. The concentration in each fraction was determined by fluorescent spectrophotometer (λ_{ex} = 480 nm, λ_{em} = 518 nm). For this, standard curves of each polymer were created using both 1-octanol/dichloromethane saturated with PBS or PBS saturated with 1-octanol/dichloromethane, respectively. The partition coefficient (P) was calculated as $P = c_{org}/c_{PBS}$; where c_{org} is polymer concentration in the organic phase (1-octanol or dichloromethane) and c_{PBS} being the polymer concentration in PBS. All measurements were performed in triplicate.

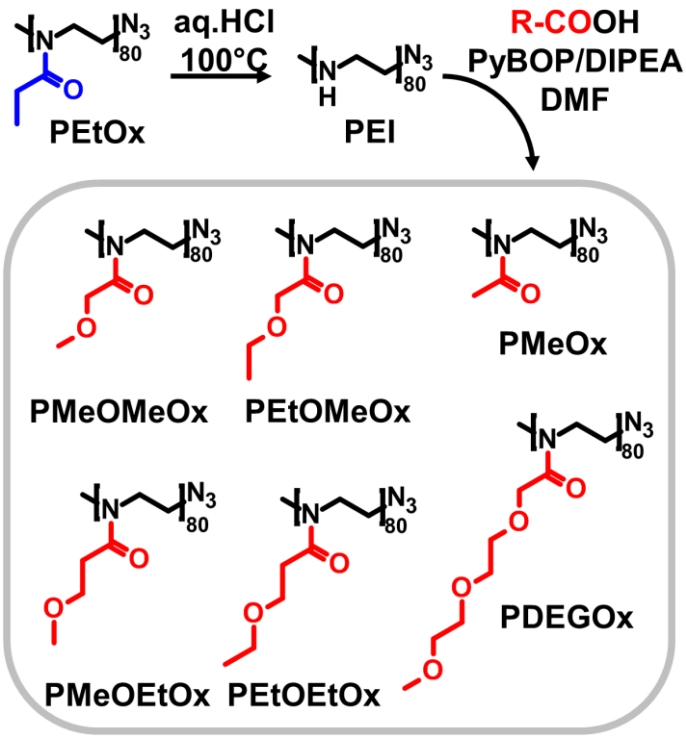
The *in vitro cytotoxicity* of the prepared PAOx was evaluated using the cervical carcinoma cell line HeLa. 5×10^4 of HeLa cells were seeded in 100 µL of media into 96-well flat-bottom TPP plates (Thermo-Fisher Scientific, Czech Republic) for 24 h before adding the polymers, of which the concentration was varied in the range 100 – 0.1 µg ml^{-1}. The cells were cultivated at 37 °C for 72 h under 5 % CO_2 atmosphere. AlamarBlue® cell viability reagent (10 µl; Thermo-Fischer Scientific) was added to each well and incubated at 37 °C for 4 h. The active component of the AlamarBlue reagent resazurin was reduced to the highly fluorescent resorufin only in viable cells. Its fluorescence was detected in a Synergy Neo plate reader (Bio-Tek; Winooski,

VT, USA) using excitation at 570 nm and emission at 600 nm. As a control, the cells cultivated in medium without PAOx were utilized. The assay was repeated two to three times in triplicate and quadruplicate.

RESULTS AND DISCUSSION

The synthetic approaches used in this work are depicted in **Scheme 1**. The starting material, a well-defined poly(2-ethyl-2-oxazoline) (PEtOx) having a degree of polymerization (DP) of 80, was synthesized by living cationic ring-opening polymerization (CROP) of EtOx in acetonitrile followed by the termination of the polymerization with sodium azide. The latter step introduces the chain-end azide group suitable for further functionalization. The obtained PEtOx was subjected to a controlled acidic hydrolysis in aqueous hydrochloric acid to yield linear PEI with a low molar mass distribution ($Đ$ = 1.09, **Figure S1**). The full conversion was confirmed by ^{1}H NMR spectroscopy, where both signals of the PEtOx side-chains completely disappeared and the main backbone ethylene signal was shifted from ~3.5 ppm (PEtOx ethylene groups adjacent to the amide) to ~2.8 ppm (PEI ethylene adjacent to amine) (**Figure 1**). The FTIR spectroscopy also revealed the disappearance of characteristic PEtOx amide carbonyl vibration at 1620 cm^{-1} (**Figure S2**) while a new strong peak appeared at 3214 cm^{-1}, which can be assigned to the N-H stretch vibration of PEI. Finally, the presence of the chain-end azide group was confirmed by its characteristic vibration at 2100 cm^{-1}.

Scheme 1. Synthesis of new PAOx *via* acylation of well-defined PEI.



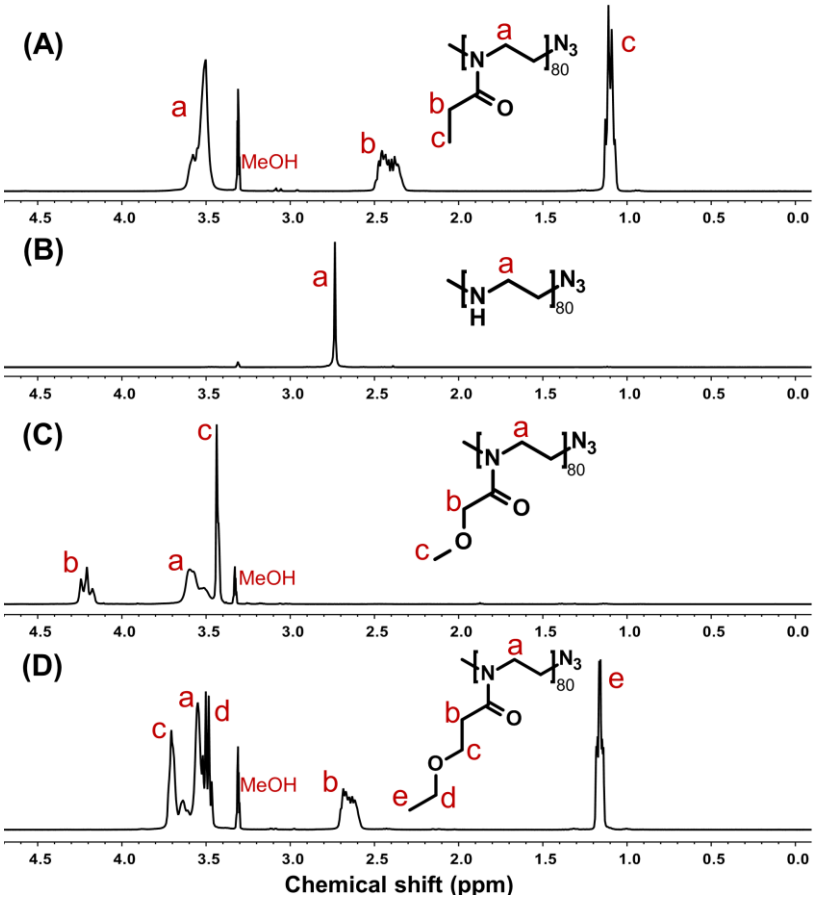


Figure 1. Representative ^{1}H NMR spectra (400 MHz) of PEtOx (A), PEI (B), PMeOMeOx (C) and PEtOEtOx (D) in CD_3OD.

For certain biomedical applications, such as non-fouling coatings and drug carriers, the high hydrophilicity and biocompatibility of new polymers is desired. Therefore, the obtained PEI was re-acylated with a series of relatively hydrophilic ether-containing carboxylic acids (**Table 1**) to obtain a library of new water-soluble PAOx. Additionally, acetic acid was used for the synthesis of the widely used hydrophilic PMeOx to prove that its properties do not differ from those of the same polymer prepared by CROP. Inspiration for the synthesis of such PAOx via acylation rather than the synthesis and polymerization of novel monomers was our previous unsuccessful attempt to synthesize the MeOEtOx monomer. During the monomer synthesis, the base-catalyzed elimination of methanol occurred, resulting in MeOEtOx heavily contaminated with 2-vinyl-2-oxazoline (**Figure S3**). The new acylation protocol overcomes this difficulty.

The PEI amidation was performed by a standard peptide coupling protocol using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling agent, DIPEA as base and dry DMF as solvent yielding the new PAOx as colorless products.[37] Note that this method was preferred over the use of acid chlorides as the latter leads to brown coloration of the polymers, presumably due to partial oxidation of the amino groups. To ensure full conversion of the secondary amines, the reaction mixture was stirred overnight. This approach is rather universal and can be used for a variety of carboxylic acids to quickly synthesize libraries of different PAOx without the need of monomer synthesis and purification, which can be difficult or time-consuming. Additionally, it can be used for the synthesis of random PAOx copolymers in cases, where different monomer reactivities cause gradient copolymer structures (e.g., MeOx with PhOx).[25] As this standard protocol can be used for coupling of amino acids with unprotected hydroxyl groups (e.g., serine), we attempted to acylate PEI with lactic acid with the aim to make a very hydrophilic poly(2-hydroxy-2-ethyl-2-oxazoline). Unfortunately, the lower reactivity of the PEI secondary amines together with the required longer reaction time resulted in the unwanted acylation of lactic hydroxyls and incomplete acylation of the polymer backbone (**Figure S4**).

Table 1. Characteristics of prepared PAOx.

Polymer	R-COOH[a]	M_w (kDa)[b]	M_n (kDa)[b]	$Đ$[b]	T_g (°C)[c]	c_p (J g^{-1}K^{-1})[c]	T_d (°C)[d]
PEtOx	-	7.6	7.1	1.07	53.2	0.51	388
PMeOx	Acetic acid	7.9	7.5	1.15	73.7	0.40	354
PMeOMeOx	MeOMeCOOH	9.0	8.3	1.10	32.4	0.56	356
PEtOMeOx	EtOMeCOOH	8.4	7.1	1.18	20.2	0.59	332
PMeOEtOx	MeOEtCOOH	8.3	7.5	1.11	21.4	0.78	334
PEtOEtOx	EtOEtCOOH	9.3	8.3	1.18	8.0	0.52	320
PDEGOx	MeOEtOEtOMeCOOH	14.7	12.4	1.18	-25.2	0.85	312

[a]Carboxylic acid used for PEI acylation. [b]Determined by SEC in methanol - sodium acetate buffer (80:20) with light scattering detection. [c]Determined by DSC from the second heating run (5 K min^{-1}).[d]Degradation temperature at 5% mass loss determined by TGA.

The obtained hydrophilic PAOx were analyzed by various methods including NMR, FTIR spectroscopy, SEC, HPLC, turbidimetry, DLS and DSC. The ^{1}H NMR spectroscopy showed the complete disappearance of PEI signal at ŭ2.8 ppm, while new peaks originating from the PAOx backbone (ŭ3.4 ï3.6 ppm) and side chains appeared (**Figure 1** and **Figure S5**). In the case of the potential signal overlap (e.g., PEtOEtOx), the polymer structure was confirmed by ^{1}H-^{1}H COSY NMR spectra **(Figure S6).** Additionally, the quantitative conversion of amines was confirmed by a Kaiser test, for which synthesized polymers were dissolved in ninhydrin solution. While the color of the PEI sample turned dark-brown after several minutes at room temperature, the color of the obtained PAOx polymers remained unchanged even after one day of heating at 50 °C (**Figure S7**). The FTIR spectroscopy revealed the disappearance of the PEI N-H vibration at 3214 cm^{-1}, while a strong band corresponding to the amide carbonyl vibration appeared at ~1630 cm^{-1} and the characteristic ñumbrellaò v observed around 1370 cm^{-1}. Furthermore, the polymers containing ether groups showed strong C-O stretching vibrations at ~1100 cm^{-1}. As many biological properties of the polymers depend on their size (e.g., renal clearance, biodistribution, cellular uptake),[38] a high uniformity of the polymer size is desired. In this work, all synthesized polymers were well defined, with low molar mas distribution ($Đ$ < 1.2), albeit slightly less defined as the parental PEtOx, withsome minor shouldering in the SEC chromatogram due to some unavoidable (at least under the used polymerization conditions) chain transfer and chain coupling side reactions,column interactions and/or the polymer fragmentation during PEtOx hydrolysis (**Figure 2**; **Table 1**). To further explore the scope of the new acylation protocol, a series of PMeOMeOx with different DP (50, 100, 200 and 400, respectively) was synthesized (**Table S2**, **Figure S8**). All polymers were of low dispersity, however the low molar mass SEC shouldering was apparent in the polymers with DP 250 and 500 polymers, presumably resulting from the main chain fragmentation during the hydrolysis of high molar mass PEtOx.[35]

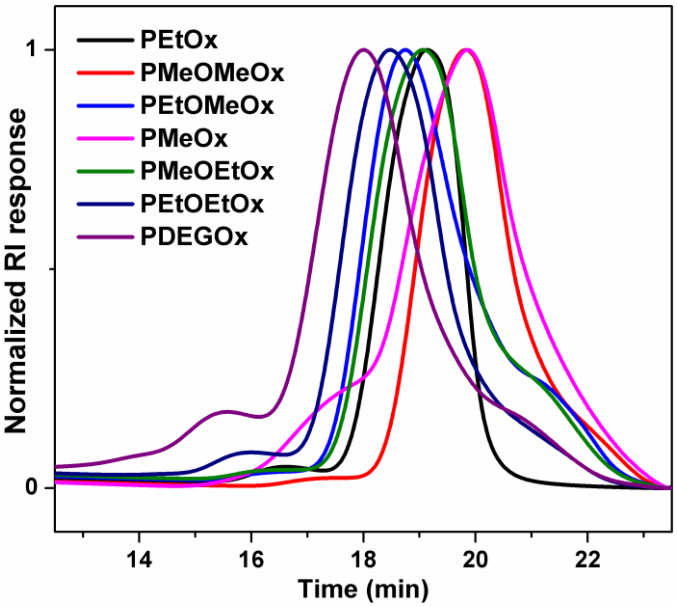


Figure 2. SEC traces of prepared PAOx polymers in methanol ïsodium acetate buffer (80:20) eluent.

The thermal properties of the new PAOx were studied by differential scanning calorimetry (DSC). All polymers exhibit amorphous behavior with glass transition temperatures (T_g) in the range from ï25 °C to 74 °C (**Table 1**, **Figure 3**). The low T_g of amorphous polymers can be beneficial in many respects as their high chain flexibility generally leads to faster dissolution. Also, the higher chain segment mobility is beneficial in the construction of various self-assembling architectures, increasing the rate of chain association/dissociation and facilitating the reproducible formation of equilibrium structures, or in the construction of magnetic resonance imaging (MRI) contrast agents, where the high chain mobility ensures fast transverse relaxation and high MRI contrast.[39] Finally, the low-T_g polymers can be used as plasticizers to improve mechanical properties of various polymer blends.[40] To date, the lowest T_g achieved within the PAOx family was reported to be ï6 °C for poly(2-(3-ethylheptyl)-2-oxazoline).[41] In the current study, the PDEGOx sample showed an even lower T_g of ï25 °C. This low T_g of PDEGOx can be explained by its structural similarity with low molar mass polyethylene glycol (PEG) that exhibits low T_g due to the low rotational activation energy of its chain segments.[42] The second polymer with a T_g significantly lower than room temperature is PEtOEtOx (T_g = 8 °C). The other ether-containing PAOx (PMeOMeOx, PMeOEtOx and PEtOMeOx) display T_g values around room temperature (20 ï32 °C; **Table 1**)

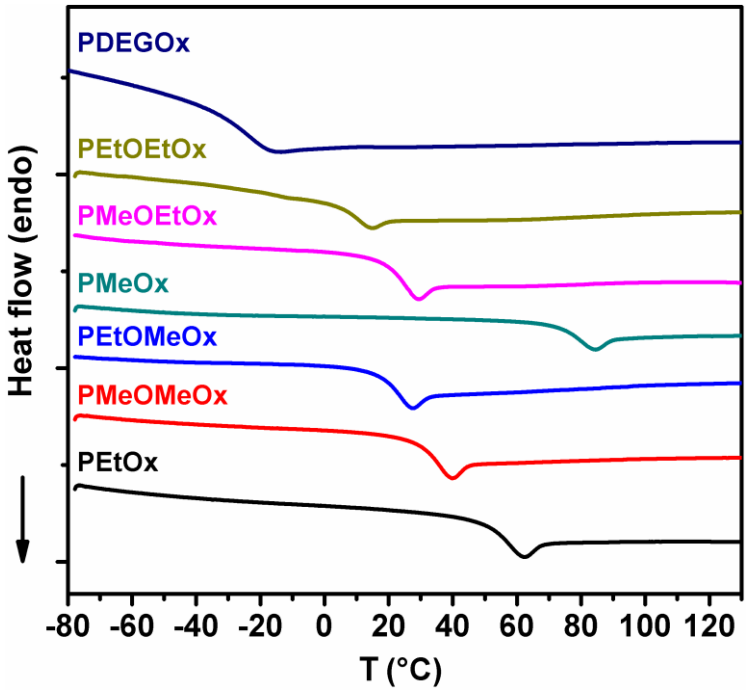


Figure 3. DSC curves of the prepared polymers.

The thermal stability of the new PAOx was determined by thermogravimetric analysis (TGA). All the polymers were stable up to a temperature of at least 300 °C (**Table 1** and **Figure S9**). With further heating, the polymers started degrading. The non-ether containing polymers (PEtOx and PMeOx), as well as the polymers containing a methylene group between the amide carbonyl and the ether (PMeOMeOx, PEtOMeOx and PDEGOx) degraded in one step. On the other side, the polymers containing ethylene group between the amide carbonyl and ether (PMeOEtOx and PEtOEtOx) degraded in two steps. The first degradation step (at ~ 320 - 335 °C) can be explained by thermal elimination of the alkoxy substituent to obtain 2-vinyl-2-oxazoline units, while the second step (at ~ 400 °C) indicates the full degradation of polymers.

All the synthesized polymers were water-soluble at room temperature with a solubility higher than 100 mg mL^{-1} (maximum concentration that was tested). As some PAOx (e.g., PEtOx, PPrOx) were reported to exhibit lower critical solution temperature (LCST) behavior in water,[43] the aqueous solubility of the newly synthesized polymers at different temperatures was studied by turbidimetry. Besides PEtOx, which shows a cloud point temperature (T_{CP}) of 89 °C (**Figure S10**), the only polymer exhibiting LCST behavior in water within the measured range (10 ï95 °C) was PEtOEtOx with a T_{CP} of 59.5 °C (**Figure 4 and S11**). The process was fully reversible with low hysteresis of 4 °C. To gain more detailed insight into its phase separation,

the self-assembly of PEtOEtOx was further investigated by dynamic light scattering (DLS) revealing a very similar value of T_{CP} of 59 °C. The LCST behavior of PEtOEtOx may be interesting for advanced thermoresponsive self-assembling architectures.

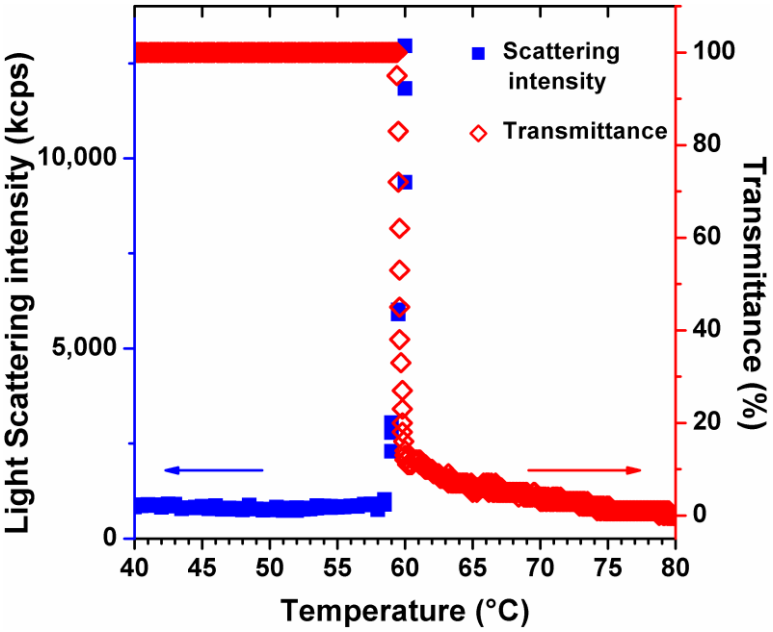


Figure 4. LCST behavior of PEtOEtOx in water (c_{pol} = 10 mg mL^{-1}) as determined by DLS (blue squares) and turbidimetry (red diamonds).

The hydrophilicity of a polymer plays a key role in its potential in biomedical applications.[44] Extremely hydrophilic polymers possess a strong hydration layer, which protects them from the unwanted interactions with blood proteins *in vivo*.[45-46] This so called "stealth" result in improved polymer pharmacokinetics as they evade plasma opsonization and clearance by mononuclear phagocytic system. Therefore, the synthesis of extremely hydrophilic polymers is desired. Herein, we assessed the relative hydrophilicity of the prepared polymers by high performance liquid chromatography (HPLC) on a reversed phase column (C18). Multi-angle light scattering detection was used as the polymers have very low UV absorption. The higher hydrophilicity of polymers is indicated by a lower retention time in HPLC due to the lower interactions with the hydrophobic column stationary phase.[16] Within the prepared PAOx series, the ether-containing polymer with the shortest side chain (PMeOMeOx) showed the highest hydrophilicity (**Figure 5, S12**), even slightly higher than PMeOx, which was considered as the most hydrophilic PAOx so far. As such, our first-time synthesis of "stealth" PMeOMeOx might provide a significant progress in the field of new biopolymers, which we will explore in future work. On the other hand, the most hydrophobic polymer from the series, PEtOEtOx, shows significant interactions with the column surface. This more

amphiphilic character of PEtOEtOx is also reflected in its abovementioned LCST behavior. Besides PMeOMeOx, PMeOx and PEtOEtOx, all prepared polymers possess a hydrophilicity that is comparable to PEG standard. Interestingly, we found a significant difference in hydrophilicity between the isostructural PMeOEtOx and PEtOMeOx, differing only in the position of the side-chain ether group. The higher hydrophobicity of PEtOxMeOx can be attributed to the closer distance of the solvated ether group to the polymer backbone, leaving more hydrophobic ethyl groups exposed for interactions with the hydrophobic column surface. This observation is in line with the previous report on lower hydrophilicity of poly(2-ethyl-2-oxazoline) compared to the isomeric poly(2-methyl-2-oxazine)s that has an additional methylene group in the main chain rather than the side chain.[47]

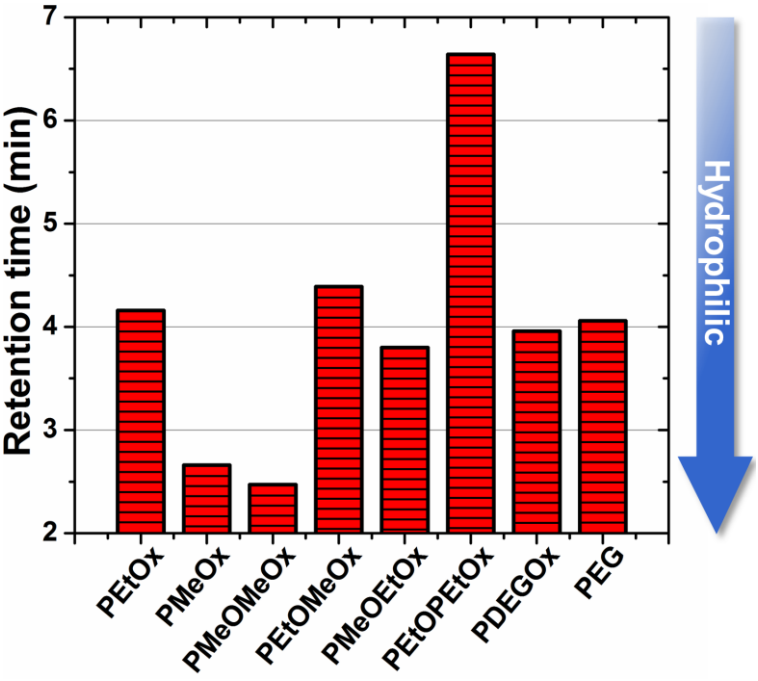


Figure 5. Reversed phase HPLC retention times of prepared PAOx and PEG.

To further investigate the hydrophilicity of the synthesized PAOx, they were conjugated with the fluorescein dye. First, the chain-end azide group was reduced with DL-dithiothreitol (DTT) in PBS. The obtained polymer chain-end amines were then functionalized with fluorescein by coupling with fluorescein isothiocyanate (FITC). The polymer-fluorescein conjugates (F-PAOx) had relatively high chain-end functionality (>60 %) and acceptable molar mass distribution (**Table S3**). Further, the fluorescently labeled polymers were used to quantitatively evaluate their hydrophilicity by measuring their distribution coefficients (*P*) between PBS and organic solvents (**Figure 6, S13**).[36] As the fluorescence intensity of fluorescein label highly

depends of pH, the polymer conjugates were dissolved in PBS (pH = 7.4) and extracted with 1-octanol, respectively dichloromethane. Surprisingly, the distribution experiments between 1-octanol and PBS suggested higher hydrophilicity of both alkyl side-chain PAOx (PMeOx and PEtOx) compared to the ether side-chain PAOx (including PMeOMeOx). This observation can be explained by the increased hydrogen bonding between the polymer ether groups and 1-octanol, resulting in increased solubility of ether side-chain PAOx in this organic phase. Therefore, 1-octanol was replaced by the non-interacting organic solvent dichloromethane to obtain unbiased measures of polymer hydrophilicity. With this method, the obtained hydrophilicities followed the similar pattern as observed in HPLC experiment, confirming that PMeOMeOx is the most hydrophilic PAOx to date, PEtOEtOx the most hydrophobic polymer from the series and PMeOEtOx being more hydrophilic that the isostructural PEtOMeOx.

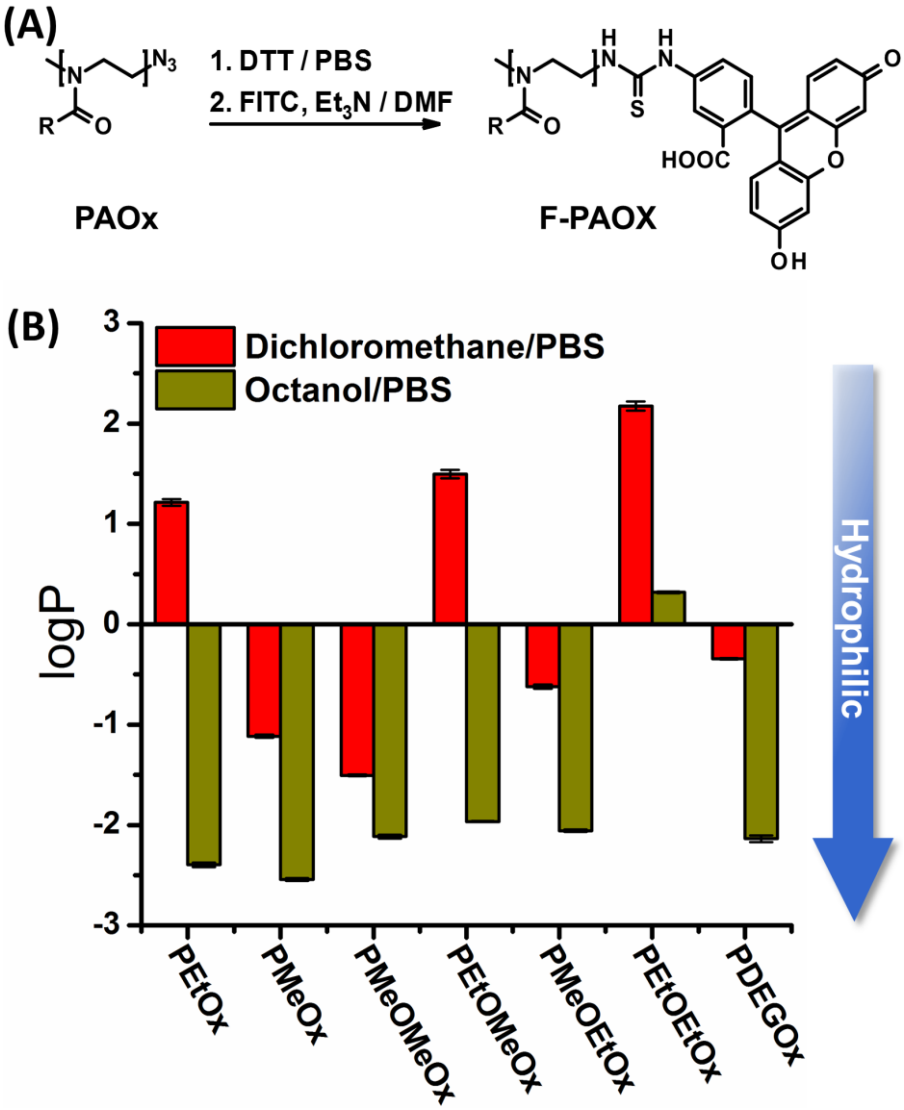


Figure 6. Labeling of PAOx with fluorescein (A) and partition coefficients of prepared conjugates (B).

The non-cytotoxic character of the synthesized PAOx was confirmed *in vitro* using an AlamarBlue® cell viability assay in cervical carcinoma HeLa cells. The polymers were incubated with cells for 76 h at 37 °C, after which the cell viability was assessed (**Figure 7**). All polymers (including PMeOMeOx) were non-cytotoxic in the concentration range of up to 0.1 mg mL^{-1} proving their excellent *in vitro* biocompatibility. In the future, detailed biological evaluation of the synthesized polymers (especially PMeOMeOx) will be performed, as they possess interesting potential for the construction of new drug/gene delivery systems and surface biocompatibilization.

Figure 7. Cytotoxicity of prepared PAOx: Dependence of HeLa cell viability on PAOx concentration (after 72 h incubation at 37 °C).

Conclusions

In summary, we developed a new method for the synthesis of functional PAOx. The easily available PEtOx is hydrolyzed to a well-defined linear PEI, which is further re-acylated with different carboxylic acids to obtain a series of new PAOx polymers. The described synthetic protocol is universal and can be used for the synthesis of PAOx libraries without the need of preparing particular 2-alkyl-2-oxazoline monomers, as well as avoiding the individual cationic ring opening polymerization procedures.