

Article

Amino-Functionalized Poly(2-Ethyl-2-Oxazoline)-Ran-Poly[2-(3-Butenyl)-2-Oxazoline] Copolymers Used as Non-Viral Vectors for Nucleic Acid Delivery: Impact of Polymer Structure and Composition

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Abstract

In this work, we designed non-viral gene delivery vector systems based on three poly(2-ethyl-2-oxazoline)-ran-poly[2-(3-butenyl)-2-oxazoline] copolymers functionalized by primary, secondary, and tertiary amino groups. The impact of copolymer structure and composition was sought through the examination of basic physicochemical and biological parameters. The complexation ability of copolymers with plasmid DNA was studied by ethidium bromide quenching assay. The polyplex particles size and ζ -potential were determined by dynamic and electrophoretic light scattering. The release ability of copolymers was assessed by competitive displacement of DNA using dextran sulfate. The biological performance of amino-functionalized poly(2-ethyl-2-oxazoline)-ran-poly[2-(3-butenyl)-2-oxazoline] based gene delivery systems was evaluated, and their behavior under various environmental conditions, such as pH and ionic strength, was investigated. Cytotoxicity was assessed in two human lung-derived cell lines, and the ability of the copolymers to mediate plasmid DNA delivery and expression was examined. The resulting polyplex nanoparticles exhibited the ability to release DNA molecules and sensitivity to alterations in pH and ionic strength. All systems showed high biocompatibility and were able to mediate plasmid DNA delivery, resulting in detectable EGFP expression in vitro. The vector properties were found to be driven by a multifactorial interplay among hydrophobic character, thermoresponsive behavior, polymer mobility, charge accessibility, intracellular environmental responsiveness, secondary structure effects, etc. The copolymer bearing primary amino groups displayed a distinct balance between DNA binding and release, characterized by moderate complex stability and enhanced sensitivity to environmental changes. These findings provide mechanistic insight into how amino functionality and polymer structure influence the structure–property–behavior relationships of polyoxazoline-based non-viral gene delivery systems.

Keywords: polyoxazolines; amino functionality; polyplexes; DNA delivery; gene expression



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1. Introduction

Over the past decades, DNA delivery has attracted significant research attention, as it enables the development of advanced therapeutic strategies [1,2]. The process is typically mediated by vectors that can condense the therapeutic nucleic acids to protect them, to facilitate the cellular uptake, to promote an efficient endosomal escape, and ultimately to enable gene expression [3,4]. Therefore, the nature, physicochemical properties, and functionality of the vector have a crucial role and are essential for effective and safe delivery of genetic material.

Polyplexes are promising non-viral gene delivery vector systems [5–7]. They represent nanosized polyelectrolyte particles formed from polymers and DNA. Since the complexation is based on electrostatic interactions with the DNA phosphate backbone, a key requirement is the presence of positively charged groups, such as amino groups, in the polymer chain. The type of the amino group could have an impact on DNA binding as well as on the biological performance of the resulting polyelectrolyte particles [8,9]. For example, the primary amino groups are permanently charged at physiological pH and have been found to possess high binding affinity, forming stable and compact complexes with DNA [10–12]. Typical representatives of polycations bearing primary amines used as non-viral vectors are poly-L-lysine [13,14] and chitosan [15]. These polymers are characterized by excellent complexation ability, forming small and compact polyplex particles. However, the strong interaction typically limits the ability to release DNA molecules and ultimately results in relatively low efficiency. In addition, polymers with primary amines are usually associated with enhanced cytotoxicity [12,16,17]. The secondary and tertiary amines protonate to varying degrees depending on the pH of the medium and are responsible for the successful endosomal escape and eventually gene expression [12,18]. Widely studied cationic polymers for gene transfection bearing secondary and tertiary amino groups are linear polyethylenimine [19] and poly(2-(dimethylamino)ethyl methacrylate) [20]. Their enhanced buffering capacity facilitates the endosomal escape of polyplexes via the “proton sponge” effect, where osmotic swelling leads to endo-lysosomal rupture and gene release [21]. However, the partial protonation of secondary and tertiary amines at physiological pH mostly leads to weaker DNA binding ability and the formation of loose complexes usually unable to enter cells [22]. Studies have shown, however, a completely opposite trend related to the type of amino group and the biological performance of the vector systems. For instance, Schubert et al. [23] synthesized a library of methacrylate-based (co)polymers functionalized with primary, secondary, and tertiary amines. The authors observed a strong impact of the type of amino functionality on the endosomal escape and thus on the transfection efficiency, following the order primary > secondary > tertiary amino groups. Obviously, the type of amino group contributes to a varying extent to the properties of polycations used as gene vectors. However, there are many other factors that could influence the physicochemical and biological properties of the polymer vector systems. It has repeatedly been shown that the polymer nature [13], architecture [8], molar mass [24,25], amount of positively charged groups and their density [25], presence of comonomers [23], hydrophobicity [26], etc., could be essential for the binding ability, size, structure, cytotoxicity and transfection efficiency of the vectors.

Polyoxazolines (POx) are a class of polymers with diverse chemical and structural versatility, functionalities, and prominent biocompatibility [27,28]. They can be synthesized via living cationic ring-opening polymerization of 2-oxazolines, offering precise control over the polymerization reaction and enabling the preparation of a wide range of well-defined polymers. An important feature of POx is that some members of their class exhibit thermoresponsive behavior, characterized by reversible soluble-to-insoluble state transition in water due to small changes in temperature [29]. The manifestation of a lower critical

solution temperature (LCST) designates them as smart materials and contributes to a wide range of additional specific properties. POx-based delivery systems have also been reported to exhibit stealth properties, significantly improving pharmacokinetics and pharmacodynamics, while demonstrating rapid clearance from the human body [30,31]. Therefore, they represent an attractive platform for the development of various biomaterials, especially in gene delivery when carrying positively charged groups [32]. The most widely used method to produce amino-functionalized POx is the partial hydrolysis [33,34]. However, the partially hydrolyzed POx contains only secondary amines due to the formation of linear polyethylenimine, limiting the properties of the vector system. Alternatively, using an appropriate synthetic strategy, POx containing primary, secondary, and/or tertiary amino groups could be produced [35–37]. Such amino-functionalized POx have recently attracted scientific attention since they can be used in various biomedical areas such as carriers of therapeutic agents, antifouling coatings, tissue engineering, etc. [35–37] but also giving rise to the development of an attractive platform for gene delivery.

Aiming at the rational design of biocompatible polymer-based gene delivery systems, it is essential to better understand how different amino functionalities influence the physicochemical and biological properties of the resulting materials, excluding the influence of additional factors such as polymer architecture, composition, etc. Therefore, a copolymer of poly(2-ethyl-2-oxazoline)-ran-poly[2-(3-butenyl)-2-oxazoline] (PEtOx-PBtOx) was synthesized and subsequently functionalized to produce three copolymers bearing primary, secondary, or tertiary amino groups in the alkyl side chain. Their interactions with DNA were investigated, assessing (i) the effects of copolymer composition on the binding and release abilities of the copolymers, (ii) the physicochemical parameters of the polyplexes at variations in the environmental conditions, and (iii) how they are related to the biological performance of the PEtOx-PBtOx-based gene delivery systems.

2. Materials and Methods

2.1. Materials

2-ethyl-2-oxazoline (EtOx, >99%, Sigma–Aldrich, Steinheim, Germany) and 2-(3-butenyl)-2-oxazoline (BtOx, 95%, Angene, London, UK) were dried over CaH_2 (95%, Sigma-Aldrich, Steinheim, Germany) and distilled into a flame-dried ampoule using a high-vacuum pump set (nEXT Turbomolecular Pumping Station equipped with an nEXT85H turbomolecular pump and an nXDS6i backing pump, Edwards Ltd., Crawley, UK). Acetonitrile (for HPLC, POCH, Gliwice, Poland) was dried over CaH_2 and distilled under a dry argon atmosphere. THF (pure for analysis, POCH, Gliwice, Poland) was dried over CaH_2 , distilled, and refluxed over a Na/K alloy. Methanol (min. 99.85%, Chemsolve Daventry, UK) for the reaction was refluxed over magnesium chips, while for dialysis, it was used without purification. DMF (for HPLC, POCH, Gliwice, Poland) was distilled under reduced pressure. Methyl 4-nitrobenzenesulfonate (99%, Sigma-Aldrich, Steinheim, Germany), p-xylene (min. 99%, VWR International, Leuven, Belgium), KOH (pure, POCH, Gliwice, Poland), cysteamine (>98%, Sigma-Aldrich, Steinheim, Germany), 2-(butylamino)ethanethiol (97%, Sigma-Aldrich, Steinheim, Germany), 2-(dimethylamino)ethanethiol hydrochloride (95%, Sigma-Aldrich, Steinheim, Germany), 2,2-dimethoxy-2-phenylacetophenone (DMPA) (99%, Sigma-Aldrich, Steinheim, Germany), benzophenone (BP) (>99%, Fluka, Buchs, Switzerland), CaCl_2 (95%, Sigma-Aldrich, Steinheim, Germany), dextran sulfate (DS, $M_w \sim 40\,000\text{ g}\cdot\text{mol}^{-1}$, Sigma-Aldrich, Merck-Bulgaria, Sofia, Bulgaria) and ethidium bromide (EtBr, Sigma-Aldrich, Merck-Bulgaria, Sofia, Bulgaria) were used as received. Plasmid DNA containing the gene encoding the enhanced green fluorescent reporter protein pEGFP-C1 (4730 bp) was isolated from a glycerin culture of *E. coli* dh 5 alpha. DNA and polymer solutions were prepared with ultra-pure water (>18 M Ω).

2.1.1. Cell Lines

Cells H1299 (human non-small cell lung carcinoma) and MRC5 (human lung fibroblasts), both sourced from ATCC (LGC Standards, Kielpin, Poland), were cultured at 37 °C in a humidified atmosphere with 95% air and 5% CO₂. H1299 cells were grown in RPMI-1640 medium (Thermo Fisher Scientific, Antisel, Sofia, Bulgaria), while MRC5 cells were cultured in Eagle's Minimum Essential Medium (Thermo Fisher Scientific, Antisel, Sofia, Bulgaria). Both media were supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Antisel, Sofia, Bulgaria) and antibiotics/antimycotics, including penicillin, streptomycin, and amphotericin B (Merck-Bulgaria, Sofia, Bulgaria).

2.1.2. Synthesis of Amino-Functionalized PEtOx-PBtOx Copolymers

The polymeric precursor was synthesized via cationic ring-opening polymerization (CROP) in a one-pot reaction. The theoretical degree of polymerization (DP) was assumed to be 70 for EtOx and 30 for BtOx. Methyl 4-nitrobenzenesulfonate dissolved in acetonitrile was placed in a reactor that had been dried under reduced pressure. EtOx and BtOx were then added under an argon atmosphere, and the reaction temperature was maintained at 70 °C. Monomer conversion was monitored by gas chromatography, with p-xylene as the internal standard. Upon complete monomer conversion, chain termination was performed by adding aqueous KOH. The final copolymer was obtained by removing acetonitrile, dissolving the residue in water, and lyophilizing. The synthesized polymeric precursor was designated as PEtOx-PBtOx. The copolymer was dissolved in a THF/methanol mixture (50:50 *v/v*, *c* = 50 mg·mL^{−1}) in a reactor that had been dried under reduced pressure. Aminothiol was then added under an argon atmosphere (for cysteamine and 2-(butylamino)ethanethiol: tenfold molar excess with respect to the vinyl groups present in the POx substituents; for 2-(dimethylamino)ethanethiol: equimolar amount). Subsequently, DMPA and BP (each equimolar to the vinyl groups of POx) were added. After complete dissolution of the reactants, the mixture was degassed and irradiated with UV light from a 2 × 15 W lamp (Uvitec, Cambridge, UK, λ = 254 nm) for 24 h. The resulting copolymer solution was then dialyzed against methanol (MWCO 1 kDa), with multiple solvent exchanges. Final dialysis was performed against water, after which the aqueous polymer solution was lyophilized. Copolymers modified with cysteamine, 2-(butylamino)ethanethiol, and 2-(dimethylamino)ethanethiol were designated as PEtOx-PBtOx-1, PEtOx-PBtOx-2, and PEtOx-PBtOx-3, corresponding to primary, secondary, and tertiary amino groups, respectively.

2.1.3. Preparation of Polyplexes

The polyplexes were prepared by mixing an appropriate volume of copolymer aqueous solutions (0.5 mg·mL^{−1}) and a DNA (0.1 mg·mL^{−1}) aqueous solution, which was mixed to give amino-to-phosphate groups ratios (N/P) of 8, 10, and 20. The preparation of polyplexes from PEtOx-PBtOx-1 and PEtOx-PBtOx-2 was performed at 25 °C, while at PEtOx-PBtOx-3 copolymer, the temperature was kept below 14 °C during complexation, followed by conditioning of the polyplex dispersion at 25 °C. The pH of all of the polymer solutions was adjusted to 7 by adding 0.1 M NaOH before polyplex preparation. For simplicity, the polyplexes formed from the PEtOx-PBtOx copolymers were noted as PEtOx-PBtOx-1/DNA, PEtOx-PBtOx-2/DNA, and PEtOx-PBtOx-3/DNA, respectively, depending on the copolymer they are formed from.

2.2. Methods

2.2.1. Gas Chromatography (GC)

Monomer conversion during CROP was monitored by GC (Shimadzu Nexis GC-2030, Kyoto, Japan) equipped with two chromatographic lines with FID and BID-2030 detectors, using a TR-5 column (30 m $\times$ 0.32 mm ID; 0.25 μ m, Thermo Scientific™, Waltham, MA, USA) and an SH-I-17 column (30 m $\times$ 0.32 mm ID; 0.50 μ m, Shimadzu, Kyoto, Japan).

2.2.2. Nuclear Magnetic Resonance (NMR)

The composition of the copolymers was analyzed by ^1H NMR spectroscopy. Spectra were recorded for samples dissolved in CDCl_3 using a Bruker Ultrashield 600 MHz spectrometer (Bruker, Billerica, MA, USA).

2.2.3. Size Exclusion Chromatography (SEC)

The molar mass and dispersity (Đ) of the copolymer precursor were determined in DMF using SEC equipped with a multi-angle laser light scattering detector (DAWN HELEOS, Wyatt Technologies, Santa Barbara, CA, USA; $\lambda = 658$ nm, LS signal) and a refractive index detector (SEC-3010 RI, WGE Dr. Bures, Dallgow, Germany; $\lambda = 620$ nm, RI signal).

2.2.4. Dynamic and Electrophoretic Light Scattering (DLS and ELS)

DLS and ELS measurements were performed on a NanoBrook 90Plus PALS instrument (Brookhaven Instruments Corporation, Nashua, NH, USA), equipped with a 35 mW red diode laser ($\lambda = 640$ nm). The determination of the hydrodynamic diameter, D_h , was carried out at a scattering angle (θ) of 90° , while the determination of the ζ -potential was carried out at a scattering angle (θ) of 15° . For ζ -potential measurements, the principle of phase analysis light scattering (PALS) was applied. Each measurement was performed in triplicate.

2.2.5. UV-Vis Spectrophotometry

Turbidimetric measurements of aqueous solutions of the copolymers (5 mg·mL $^{-1}$) were carried out using a Specord 200 plus UV-Vis spectrophotometer (Analytik Jena, Jena, Germany). The cloud point temperature (T_{CP}) was determined by the transmittance of the copolymer solutions, which reached 50% of the initial value in a temperature range within 7–65 $^\circ\text{C}$.

2.2.6. Fluorescent Spectrophotometry

The fluorescence of samples labeled with EtBr was measured at $\lambda_{\text{ex}} = 535$ nm and $\lambda_{\text{em}} = 600$ nm on an Agilent Cary Eclipse fluorescence spectrometer (Agilent Technologies, Santa Clara, CA, USA) using a quartz cell with a path length of 1 cm. The excitation and emission slit widths were set to 5 nm.

2.2.7. Buffering Capacity

The buffering capacity of copolymers was determined by standard acid-base titration. Aqueous copolymer solutions (0.5 mg·mL $^{-1}$) were prepared, and the pH was adjusted to 10 by using 0.1 M NaOH. 3 mL volume samples were titrated with 10 μ L aliquots of 0.1 M HCl down to pH 3. Pure water was titrated in parallel as a control sample. The titration was performed at 25 $^\circ\text{C}$ except for the PEtOx-PBtOx-3 copolymer solution, where the temperature was kept below 14 $^\circ\text{C}$. The buffer capacity was determined by the reciprocal value of the slope of the curves over a pH range from 10 to 3.

2.2.8. Ethidium Bromide Quenching Assay (EtBrQA)

For EtBrQA, DNA was labeled with EtBr at a molar ratio $[\text{EtBr}] = [\text{P}]/4$ ($[\text{P}]$ is the concentration of DNA phosphate groups). The stained DNA solution was used for the preparation of polyplexes at 25 °C, except for PEOx-PBtOx-3 copolymer, where the temperature was kept below 14 °C. The EtBr quenching was followed by fluorescence spectroscopy.

2.2.9. DNA Release

DNA release was evaluated by a competitive displacement by dextran sulfate (DS). Polyplexes were formed using the stained DNA solution described in Section 2.2.8. Then the DS aqueous solution ($2 \text{ mg} \cdot \text{mL}^{-1}$) was added to the polyplexes dispersion at an amount corresponding to the amount of DNA in the complex. The dispersion was stirred for 30 min, then the fluorescence of EtBr was measured, and DNA release was determined.

2.2.10. Cytotoxicity Test

The cytotoxicity of the tested copolymers and their corresponding polyplexes was evaluated using the MTT assay [38]. H1299 and MRC5 cells were seeded at 4.5×10^3 cells per well in 96-well flat-bottom plates (Corning Costar, FOT Ltd., Sofia, Bulgaria). After 24 h of incubation, cells were treated with serial dilutions of micelles and micellplexes ranging from 8 to $128 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ (based on micelle concentration in the complexes). Following a 72 h incubation at 37 °C and 5% CO_2 , the medium was replaced with phenol red-free medium containing $0.5 \text{ mg} \cdot \text{mL}^{-1}$ MTT (Invitrogen, Antisel, Sofia, Bulgaria), and cells were incubated for an additional 2.5 h under the same conditions. The assay is based on the reduction in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble purple formazan crystals by NAD(P)H-dependent cellular oxidoreductases in viable cells. Formazan crystals were solubilized with 100 μL DMSO per well, and absorbance was measured at 570 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Antisel, Sofia, Bulgaria). Data were analyzed using GraphPad Prism v.8 (Dotmatics, San Diego, CA, USA).

2.2.11. Transfection

A total of 8.5×10^3 H1299 cells were seeded per well in 96-well plates (Corning Costar Flat Bottom Cell Culture Plate, FOT Ltd., Sofia, Bulgaria). After 24 h, cells were transfected with 0.5 μg pEGFP-C1 ($0.005 \text{ mg} \cdot \text{mL}^{-1}$) using Turbofect (Thermo Fisher Scientific, Antisel, Sofia, Bulgaria) according to the manufacturer's instructions, or treated with polyplexes containing the same plasmid at a final DNA concentration of $0.01 \text{ mg} \cdot \text{mL}^{-1}$ (1 μg per well) for 6 h. EGFP-C1 expression was assessed 24 h post-transfection or treatment using a Zeiss Axiovert 200 M microscope equipped with a $10\times$ PlanApochromat objective (NA = 0.45, Zeiss, Jena, Germany) and a CCD camera (AxioCam MRm, Zeiss, Jena, Germany). At least three distinct fields per condition were captured for analysis. Images were acquired under identical settings and processed with the Fiji software package v2.14.0.

2.2.12. Statistical Analysis

One-way ANOVA followed by Dunnett's multiple comparisons test was performed to compare the mean normalized transfection efficiency of each treatment group with that of the control group (Turbofect). Statistical significance was assigned at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$.

3. Results

3.1. Synthesis and Characterization of Amino-Functionalized PEtOx-PBtOx Copolymers

The focus of the current study require to obtain POx functionalized with primary, secondary, and tertiary amino groups. Copolymers based on 2-ethyl-2-oxazoline (EtOx) and 2-(3-butenyl)-2-oxazoline (BtOx) were synthesized. In order to obtain biocompatible gene delivery systems, the targeted molar mass of copolymers was below $15,000 \text{ g} \cdot \text{mol}^{-1}$ and the amount of the amino groups was below 15 mol%. Firstly, a polymeric precursor with a random microstructure composed of EtOx and BtOx units was synthesized via CROP. The reaction scheme is presented in Figure 1. The composition of the resulting copolymer was determined by ^1H NMR (Figure 2a) and was consistent with the feed ratio EtOx:BtOx = 73:27 mol%. The molar mass of PEtOx-PBtOx copolymer was determined by SEC and was in good agreement with the theoretical value calculated from the monomer feed ($M_n = 11,700 \text{ g} \cdot \text{mol}^{-1}$, $D = 1.07$). A monomodal peak with narrow molar mass dispersity was observed, as can be seen in Figure S1.

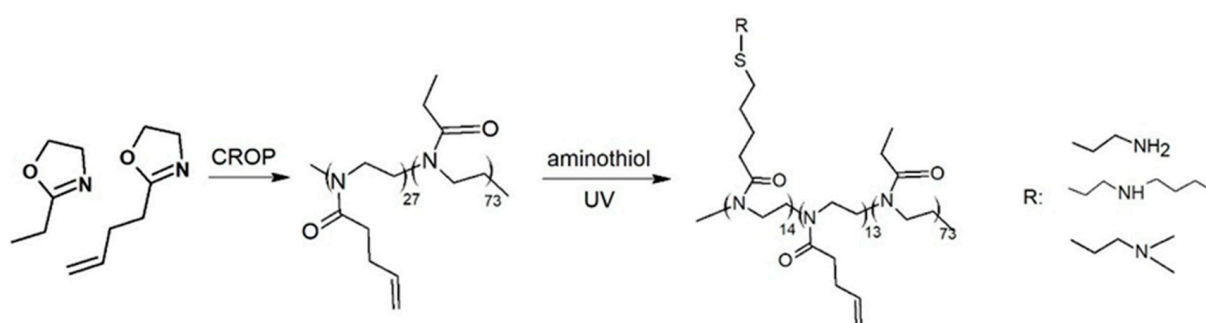


Figure 1. Synthesis of the copolymer composed of EtOx and BtOx via CROP and its subsequent post-polymerization modification through thiol-ene click chemistry using primary, secondary, and tertiary amines.

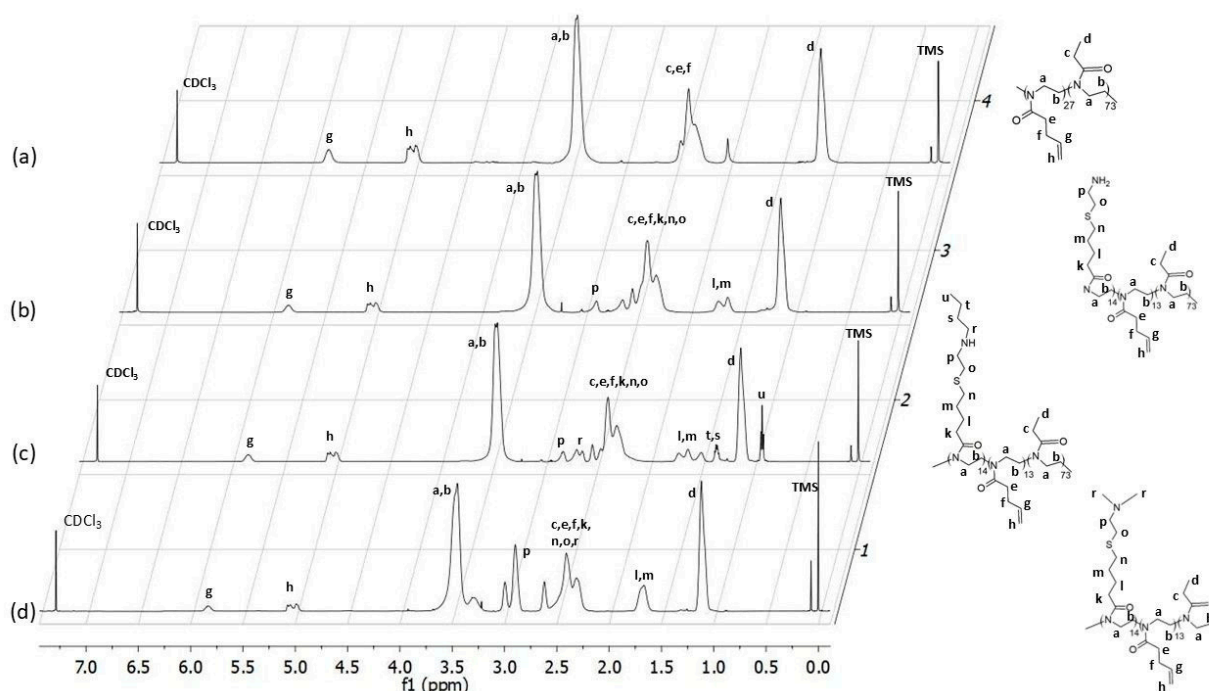


Figure 2. ^1H NMR spectra (600 MHz, CDCl_3) of the polymeric precursor composed of EtOx and BtOx (a), and its derivatives bearing primary (b), secondary (c), and tertiary (d) amino groups.

Next, the resulting copolymer was further modified via radical addition of the appropriate amino thiol onto the vinyl double bonds of the BtOx substituents by thiol-ene click reaction, as shown in Figure 1. The copolymer modification was confirmed by ^1H NMR from the decrease in the signals from protons of methylene groups of BtOx at 4.95–5.10 ppm and protons of methine groups of BtOx at 5.75–5.90 ppm (Figure 2b–d). Thereby, PEtOx-PBtOx copolymers containing 14 mol% of units substituted with pendant amino groups were obtained with an efficiency of substitution of approx. 50%. For simplicity, the copolymers functionalized with primary, secondary, and tertiary amino groups were named PEtOx-PBtOx-1, PEtOx-PBtOx-2, and PEtOx-PBtOx-3, respectively. Their molar masses were determined by SEC (see Figure S1), and the values are summarized in Table 1.

Table 1. Molar masses, cloud point temperature (TCP), and ζ -potential of PEtOx-PBtOx copolymers functionalized with amino groups.

Polymer	Amine Group	Molar Mass *, $\text{g} \cdot \text{mol}^{-1}$	T_{CP} , $^{\circ}\text{C}$	ζ -Potential, mV
PEtOx-PBtOx-1	1 $^{\circ}$	11,700	50	25.9
PEtOx-PBtOx-2	2 $^{\circ}$	12,500	14	20.6
PEtOx-PBtOx-3	3 $^{\circ}$	12,100	-	22.8

* determined from ^1H NMR.

The thermoresponsive properties of the resulting amino-functionalized copolymers were evaluated by standard UV-Vis spectroscopy and the turbidity method. Commonly, by reaching changes in light transmittance through aqueous solution with increasing temperature, the cloud point temperature (T_{CP}) can be determined. The transmittance vs. temperature curves are given in Figure S2. The results indicate a lack of thermoresponsiveness for the PEtOx-PBtOx-3 copolymer, while at PEtOx-PBtOx-1 and PEtOx-PBtOx-2, a T_{CP} was detected (Table 1). The different thermoresponsive behavior of PEtOx-PBtOx copolymers was obviously a function of the hydrophobicity of the alkyl chain of the pendant amino groups. Furthermore, the presence of amino groups provokes positive values of ζ -potential of all PEtOx-PBtOx copolymers ranging from 20.6 to 25.9 mV (Table 1).

The presence of amino groups in the copolymer structure is a prerequisite for its capability to respond to changes in pH. The latter has an impact on the polymer vector traffic and endosomal recycling due to different extracellular and intracellular pH environments. The buffering capacity of the amino-functionalized PEtOx-PBtOx copolymers was estimated by standard acid-base titration. The curves giving the variations in pH over the 10 to 3 range are presented in Figure 3a. Pure water was titrated as a control sample. The buffering capacity was quantified from the reciprocal value of the slope of the titration curves in the same pH range and is given in Figure 3b. It was evident that, despite the low content of amino groups (14 mol%), all copolymers showed buffering capacity. Quite expected, the copolymers possessing secondary and tertiary amino groups (PEtOx-PBtOx-2 and PEtOx-PBtOx-3) exhibited a higher ability to protonate than the copolymer with primary amines.

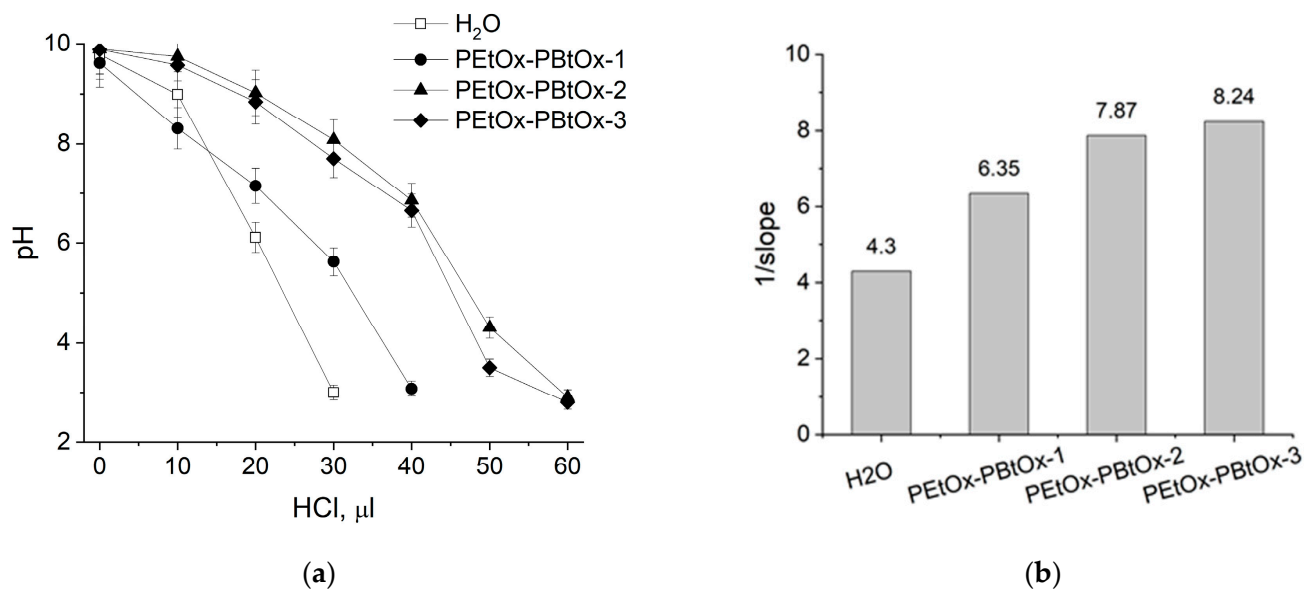


Figure 3. Buffering capacity of amino-functionalized PEOx-PBtOx copolymers functionalized with amino groups given as variations in pH vs. HCl volume (a) and reciprocal values of the slope of curves (b). The presented data include the mean $\pm$ SD of three replicates.

3.2. Complexation with DNA

The ability of the resulting amino-functionalized PEOx-PBtOx copolymers to form complexes with DNA was investigated. Copolymers and DNA aqueous solutions were mixed to prepare polyplexes at an amino-to-phosphate group (N/P) ratios of 8, 10, and 20. The polyplexes of PEOx-PBtOx-1 and PEOx-PBtOx-3 were prepared at 25 $^{\circ}\text{C}$, whereas those of PEOx-PBtOx-2 were prepared at a temperature below 14 $^{\circ}\text{C}$ to ensure that the copolymer is molecularly dissolved. The binding ability of the PEOx-PBtOx copolymers was studied using dye-labeled DNA. EtBr is an intercalating agent commonly used as a fluorescent tag for DNA staining [39]. The quenching of EtBr fluorescence is a sign of complex formation between the copolymer and DNA. As evident from Figure 4, even at an N/P ratio of 8, a significant drop in EtBr fluorescence, ranging between 37 to 20%, was observed. A further increase in the amount of copolymer led to an additional decrease in fluorescence intensity, and at an N/P ratio of 20, almost all of the dye was displaced, indicating the strong complexation ability of PEOx-PBtOx copolymers. Nevertheless, an effect of the type of amino group was detected. Unexpectedly, PEOx-PBtOx-1 exhibited a lower ability to displace the EtBr in contrast to PEOx-PBtOx-2 and PEOx-PBtOx-3. According to the literature, primary amines have better complexation ability than the secondary and tertiary ones [10,12]. Therefore, a possible explanation could be found not in the type of the amino group but in the length of the pendant alkyl chain. Studies revealed that the longer the hydrophobic side chain, the better the DNA condensation [26]. Referring to the different hydrophobicity of the synthesized POx copolymers (Figure 1 and Table 1), it is obvious that the length of the pendant alkyl chain has a competitive impact on the complexation ability of the copolymers together with the type of the amino group.

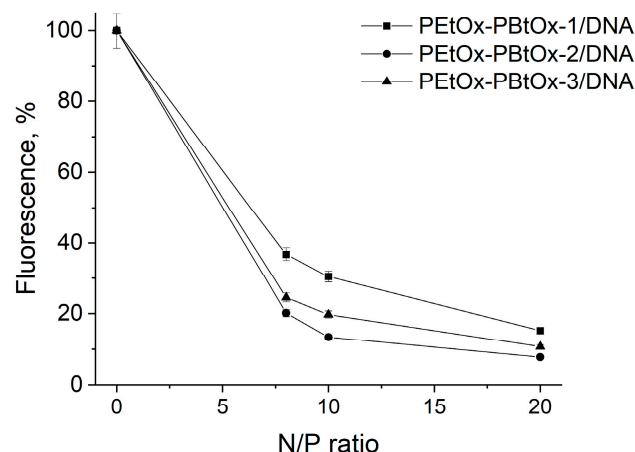


Figure 4. Variations in EtBr fluorescence in polyplexes formed from amino-functionalized PETox-PBtOx copolymers functionalized with amino groups and plasmid DNA.

The polyplex particles were characterized with respect to their sizes and surface potential. In Figure 5a, the variations in the hydrodynamic diameter, D_h , determined by dynamic light scattering, are shown. All particles obtained were in the sub-100 nm scale, independently from the polymer composition and the N/P ratio, meeting the size requirements for efficient cellular internalization [40]. It was noteworthy that PETox-PBtOx-2 copolymer formed smaller polyplex particles (c.a. 78 nm) barely influenced by the N/P ratio. The D_h of PETox-PBtOx-3/DNA was slightly higher c.a. 90 nm, at N/P ratios of 8 and 10, compared to PETox-PBtOx-2/DNA and decreased to 78 nm at N/P 20. In general, PETox-PBtOx-1 copolymer formed larger polyplexes, however, a well pronounced effect of the N/P ratio was observed. These complexes shrank in size upon increasing the copolymer content—100 vs. 94 vs. 88 nm at N/P ratios of 8, 10, and 20, respectively. The size variations can be related to the binding ability of copolymers observed from EtBrQA (Figure 4). The larger size and lower binding of PETox-PBtOx-1 copolymer may imply formation of somewhat looser polyplexes. In a previous study, a similar behavior of POx functionalized with pendant primary and secondary amines has been reported [37]. The copolymers bearing primary amines formed bigger polyplexes than the copolymers functionalized with secondary amino groups. The authors allowed for the formation of hydrogen bonds when pendant primary amines are present, competing the DNA condensation [37]. POx are known as pseudo peptides due to the similarities in their chemical structures [28,29], therefore, the formation of pseudo peptide intramolecular hydrogen bonds in the case of PETox-PBtOx-1 copolymer could be suggested. The latter probably contributed to the weaker DNA binding and the formation of less compact polyplex particles compared to those formed from PETox-PBtOx-2 and PETox-PBtOx-3 copolymers. As expected, the ζ -potential exhibited entirely positive values ranging from 18 to 23.1 mV (Figure 5b). The complexes formed from PETox-PBtOx-1 copolymer were somewhat more positive than PETox-PBtOx-2/DNA and PETox-PBtOx-3/DNA. The latter was in agreement with the ζ -potential values determined for the neat copolymers (Table 1).

The polyplex particles were visualized with cryo-TEM. In Figure S3, representative micrographs as well as size distributions observed from cryo-TEM are shown. As is evident, the complexes represent well-defined spherical particles with an average size in the 84–116 nm range. Separate fractions of smaller particles were detected however, all polyplexes were characterized with good size distribution without the presence of aggregates.

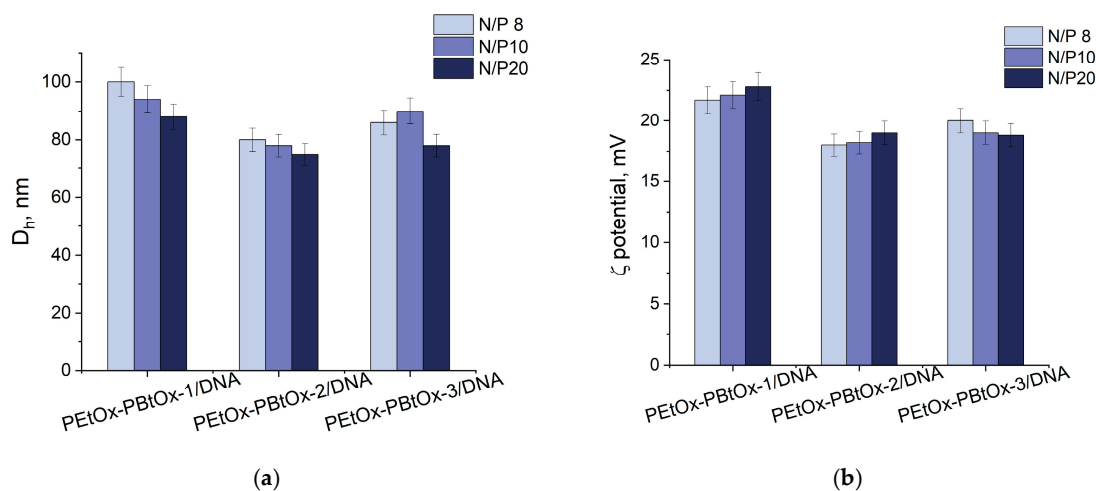


Figure 5. Variations in hydrodynamic diameter, D_h , (a) and ζ -potential (b) of polyplexes formed from PETox-PBtOx copolymers functionalized with amino groups and plasmid DNA.

3.3. DNA Release and Effect of Environmental Conditions

A competitive DNA displacement by DS was used to study the release ability of the PETox-PBtOx copolymers. DS is a strong polyanion that is known to have the ability to displace the DNA from its interactions with positively charged species such as proteins, cationic particles, molecules, etc. [41,42]. Therefore, the addition of DS to the polyplex dispersion could serve for the evaluation of the copolymer's ability to release DNA. DNA labeled with EtBr was used for this experiment. The release was assessed by the increased fluorescence intensity of the dye after displacement of DNA (Figure S4). In Figure 6, the DNA release is given as a percentage of the recovered fluorescence intensity. As is evident, in all systems, a free DNA was detected with an amount dependent on the copolymer used and the N/P ratio. Quite expected, increasing the amount of the copolymer tends to decrease the release ability. In general, the % of released DNA was the highest for the PETox-PBtOx-1 copolymer. This copolymer exhibited up to 63% released DNA at an N/P ratio of 8. In contrast, PETox-PBtOx-2 and PETox-PBtOx-3 copolymers released about 50% of DNA at the same N/P ratio. The increased release ability of the PETox-PBtOx-1 copolymer could be attributed to its weaker DNA binding and the formation of looser complexes compared to PETox-PBtOx-2 and PETox-PBtOx-3 copolymers. The obtained data outline the N/P 8 as the optimal ratio with respect to the release ability. Additionally, at this N/P ratio, the amount of polycation in the polyplexes composition is lower than at N/P ratios 10 and 20, assuming their better cytocompatibility. Therefore, our further experiments were performed at this N/P ratio.

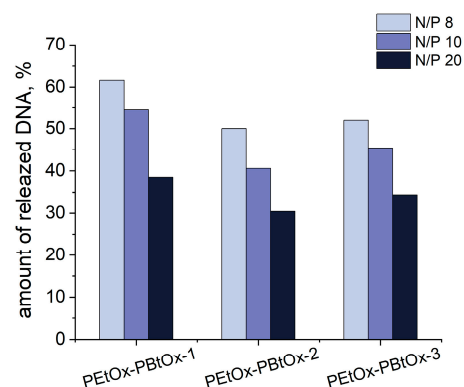


Figure 6. Release ability of PETox-PBtOx copolymers estimated by a competitive DNA displacement by dextran sulfate (DS).

The traffic of vector systems inside the cells is associated with abrupt changes in the environmental conditions. For example, the extracellular pH in the human body is in the 7.35–7.45 range, characterized by an ionic strength ranging from 0.13 to 0.15 M. Entering the cells, the polyplexes fall into endosomes where the pH decreases to app. 6.0–6.5 in early and 5.0–5.5 in late. Then the endosomes mature into lysosomes, characterized by a more acidic pH ranging from 4.5 to 5.0 [43]. Therefore, the ability of polyplex particles prepared from PEtOx-PBtOx copolymers to respond to changes in ionic strength and pH of the medium was studied. In Figure 7, the variations in D_h and ζ -potential in the presence of 0.15 M NaCl as well as at pH 4, are given. First, the polyplexes were exposed to conditions resembling the cell culture medium—pH 7.4 and 0.15 M NaCl (violet symbols). Under these conditions, all the systems undergo changes in D_h and ζ -potential. Their size increased, while the ζ -potential sharply turned into negative values. Control experiments performed at the same ionic strength but without changing pH (green symbols), as well as in the absence of NaCl at pH 7.4 (red symbols), reveal that the changes observed are salt-induced. This behavior is usually associated with competitive complex formation between the phosphate groups of DNA and sodium ions. The latter caused polyplexes destabilization and swelling, accompanied by a reduction in intra-chain charge repulsions, giving rise to negative values of ζ -potential [44]. It can be seen, however, that the increase in particle size was less pronounced for PEtOx-PBtOx-1/DNA, 2.5 times compared to 5 times for PEtOx-PBtOx-2/DNA and 3.7 times for PEtOx-PBtOx-3/DNA. This could be attributed to the formation of pseudo-peptide intramolecular hydrogen bonds at this copolymer that are known to strengthen hydrophobic interactions, thus enhancing the stability of the peptide structure. The polyplexes were also exposed to acidic pH (blue symbols). Surprisingly, the particles formed from PEtOx-PBtOx-2 and PEtOx-PBtOx-3 copolymers (bearing secondary and tertiary amino groups) were barely influenced by the pH. This could be due to their better binding ability (Figures 4 and 5), which is enhanced in acidic media due to the protonation of the secondary and tertiary amino groups, giving rise to stronger interaction with DNA [44]. In contrast, an increase in size was observed for PEtOx-PBtOx-1/DNA accompanied by a slight decrease in their ζ -potential at pH 4. It was supposed that this observation was also related to the presence of intramolecular hydrogen bonds provoking pseudo-peptide behavior of the PEtOx-PBtOx-1 copolymer. It is well known that the pH is a key factor for peptides affecting their folding and stability. Thus, decreasing the pH results in the disruption of hydrogen and ionic bonds, causing the unfolding of peptide molecules. Similarly, the decrease in pH influences the strength of hydrophobic bonds in PEtOx-PBtOx-1 copolymer. This affects polymer chain flexibility, leading to changes in the conformation state to a more extended one, causing an increase in polyplexes size. The latter could be related to the beginning of polyelectrolyte complex disintegration process.

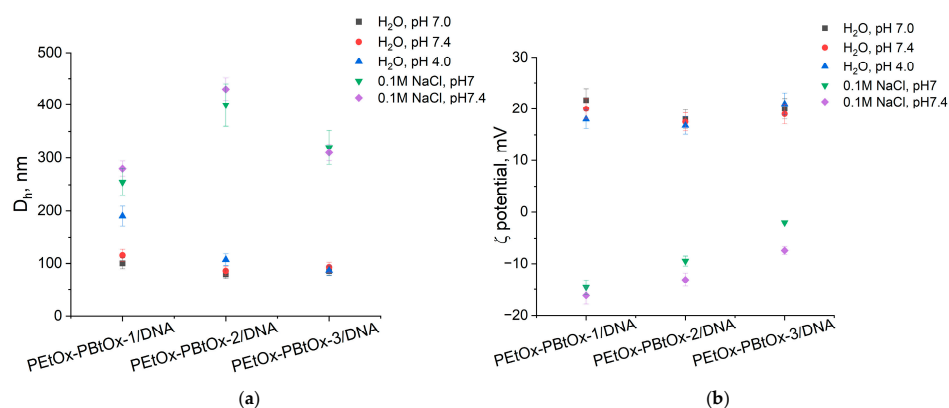


Figure 7. Variations in hydrodynamic diameter, D_h , (a) and ζ -potential (b) of polyplexes prepared at a N/P ratio of 8 from PEtOx-PBtOx copolymers under changes in pH and ionic strength of the medium.

3.4. Cytotoxicity Profile of PEtOx-PBtOx Copolymers and Polyplexes

All three copolymers exhibited excellent biocompatibility in cancerous (H1299) and non-cancerous (MRC-5) cells, with IC_{50} values exceeding $100 \mu\text{g}\cdot\text{mL}^{-1}$ (Table 2, Figure S5). These observations completely align with previous reports demonstrating the favorable cytocompatibility of POx-based materials [30–32]. Notably, PEtOx-PBtOx-2 displayed IC_{50} exceeding $5 \text{ mg}\cdot\text{mL}^{-1}$, particularly in MRC-5 cells, a value significantly different from those observed for PEtOx-PBtOx-1 and PEtOx-PBtOx-3 copolymers. This markedly reduced toxicity may be associated with the low T_{CP} of PEtOx-PBtOx-2 (14°C , see Table 1) that favors a self-assembly into mesoglobules under physiological conditions. Such supramolecular rearrangement has been shown to attenuate cellular toxicity by decreasing polymer–membrane interactions and reducing effective charge exposure [45]. Although the complexes were formed at a low temperature, ensuring the molecularly dissolved state of the copolymer, a similar behavior was observed for the corresponding polyplexes. Interestingly, PEtOx-PBtOx-1 and PEtOx-PBtOx-3 copolymers, as well as their corresponding polyplexes, exhibited comparable toxicological profiles despite the commonly reported trend of superior cytotoxicity for primary vs. tertiary amines [46]. In the present systems, the relatively high tolerability of PEtOx-PBtOx-1 copolymer may be related to the participation of pendant primary amino groups in intramolecular hydrogen bonding, which could limit the effective electrostatic interactions with the cell membrane. The results indicate that cytocompatibility in the present POx polymer platforms is not governed solely by the amine type but rather by an interplay of several structural parameters, including hydrophobicity, thermoresponsive assembly, charge accessibility, and secondary intramolecular interactions. This highlights amino-functionality and hydrophobicity tuning as powerful design parameters for improving safety in polymer-based non-viral vectors.

Table 2. Cytocompatibility of PEtOx-PBtOx copolymers and their corresponding polyplexes. The IC_{50} values [$\mu\text{g}\cdot\text{mL}^{-1}$] are presented as mean $\pm$ SD, calculated from four technical replicates across two independent experiments. The corresponding dose–response toxicity profiles are shown in Figure S5.

	$IC_{50} \mu\text{g}\cdot\text{mL}^{-1}$			
	H1299		MRC5	
	Copolymers	Polyplexes	Copolymers	Polyplexes
PEtOx-PBtOx-1	152.6 ± 2.9	100.3 ± 1.1	159.6 ± 4.6	101.0 ± 0.2
PEtOx-PBtOx-2	801.3 ± 28.6	1057.0 ± 50.7	5282.0 ± 54.3	2305.0 ± 46.6
PEtOx-PBtOx-3	115.0 ± 1.7	102.0 ± 1.3	124.0 ± 0.9	139.3 ± 6

3.5. Transfection Efficiency and Intracellular Release Behavior

The transfection efficiency is a frequently used indicator of the relative ability of polymers to deliver nucleic acids in vitro. Therefore, the transfection efficiency of the investigated amino-functionalized PEtOx-PBtOx copolymers was studied, determining the expression level of green fluorescent protein. All vector systems successfully mediated plasmid DNA delivery into H1299 cells, resulting in detectable EGFP expression. As shown in Figure 8 PEtOx-PBtOx-1/DNA exhibited the highest transfection efficiency under the investigated conditions, while PEtOx-PBtOx-2/DNA and PEtOx-PBtOx-3/DNA displayed lower and comparable levels of expression. Notably, the transfection efficiencies of all PEtOx-PBtOx-based systems remained substantially lower than that of the commercial transfection reagent Turbofect; nevertheless, we were intrigued by the distinctions in the individual amino-functionalized PEtOx-PBtOx copolymer performance. The observed differences in transfection may be related to variations in DNA binding strength, release behavior, and physicochemical responsiveness to environmental stimuli. Among the three copolymers, PEtOx-PBtOx-1 exhibits higher release

ability and a more sustainable destabilization profile in the presence of ions. Additionally, this copolymer forms looser polyplex structures sensitive to pH variations, as evidenced by swelling behavior observed under acidic conditions. Such pH-dependent structural changes are consistent with a destabilization process that may occur along the endosomal–lysosomal pathway. While no direct evidence of endosomal escape was obtained in this study, the observed physicochemical behavior of PEtOx-PBtOx-1/DNA resembles features reported for pH-responsive peptide-based delivery systems, including amphipathic peptides such as GALA [47]. These similarities suggest a potential contribution of pH-triggered structural rearrangements to intracellular DNA availability. In contrast, the reduced response to changes in pH of PEtOx-PBtOx-2/DNA and PEtOx-PBtOx-3/DNA taken together with their weaker DNA release ability, may result in prolonged intracellular retention of compact complexes, potentially exposing DNA to nuclease degradation, ultimately limiting expression efficiency [26]. Thus, the enhanced transfection activity of PEtOx-PBtOx-1 copolymer can be attributed to a more balanced interplay between DNA protection and release, associated with moderate binding strength, and enhanced responsiveness to environmental changes, rather than to inherently high transfection efficiency.

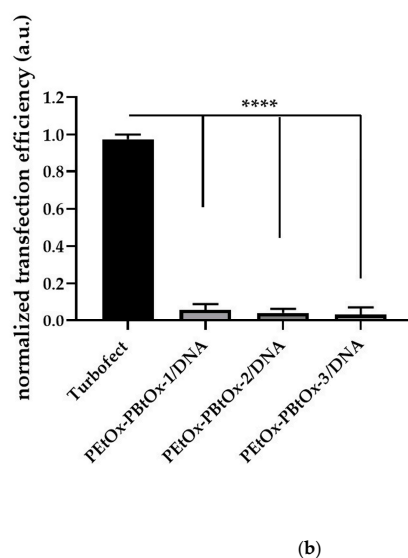
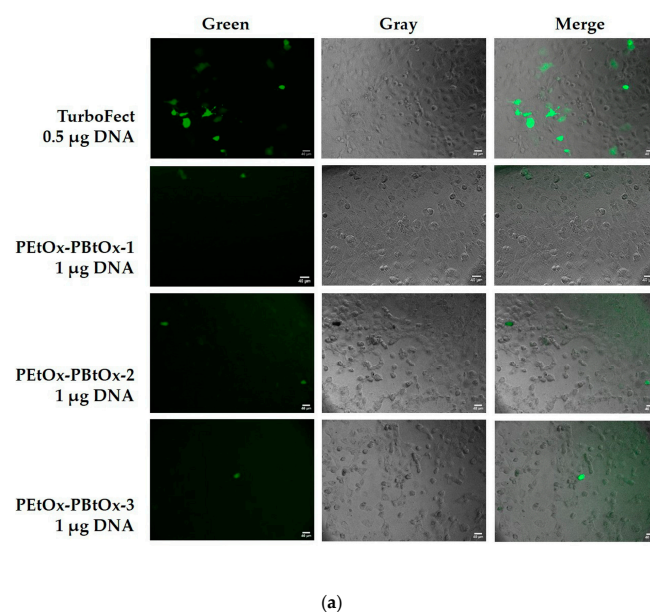


Figure 8. Functional DNA delivery and expression efficiency of PEtOx-PBtOx polyplexes. (a) Representative fluorescence microscopy images of H1299 cells transfected with pEGFP-C1 either

using Turbofect (positive control) or polyplexes prepared from the three copolymers (final DNA dose: 1 μg per well; exposure time: 6 h). Images were acquired 24 h post-treatment under identical imaging conditions. Scale bar: 40 μm . (b) Quantification of transfection efficiency is expressed as the percentage of EGFP-positive cells relative to the total cell count. Values are normalized to Turbofect (set to 100%). Data represent mean $\pm$ SD from at least two independent experiments with ≥ 3 fields analyzed per condition. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple-comparison test to compare each treatment group with the positive control (Turbofect). Normality and variance homogeneity were verified before analysis. A p -value < 0.001 (****) was considered statistically significant.

4. Conclusions

A well-defined low molar mass PEtOx-PBtOx copolymer ($11,700 \text{ g} \cdot \text{mol}^{-1}$) was synthesized via CROP. The thiol-ene click reaction was subsequently performed to produce three copolymers functionalized with primary, secondary, and tertiary pendant amino groups. Alternations in the length of the alkyl side chain resulted in PEtOx-PBtOx copolymers with different hydrophobicity and hence different thermoresponsive behavior. The resulting copolymers are characterized by positive ζ potential and buffering capacity dependent on the type of amino functionality. Despite the low content of amino groups (14 mol%), the functionalized PEtOx-PBtOx copolymers were able to condense plasmid DNA, forming sub-100 nm polyplex particles. A strong complexation ability was observed for PEtOx-PBtOx-2 and PEtOx-PBtOx-3 copolymers, while formation of looser complexes in the case of PEtOx-PBtOx-1 due to weaker binding ability was detected. This behavior was tentatively attributed to the presence of intramolecular hydrogen bonding within the PEtOx-PBtOx-1 copolymer, resembling pseudo-peptide-like interactions. The phenomenon also contributes to increased DNA release ability compared to PEtOx-PBtOx-2 and PEtOx-PBtOx-3 copolymers, as well as to distinguished sensitivity upon variations in environmental conditions, such as pH and ionic strength. Under acidic conditions, the PEtOx-PBtOx-1/DNA polyplexes underwent destabilization, consistent with disruption of intramolecular hydrogen bonding and accompanying changes in polymer chain conformation, analogous to unfolding processes described for peptide-based systems. Conversely, increasing ionic strength appeared to stabilize PEtOx-PBtOx-1/DNA, likely due to reinforcement of hydrophobic interactions associated with intramolecular hydrogen bonding. While no direct evidence of endosomal membrane perturbation or endosomal escape was obtained in this study, the observed environmental responsiveness of PEtOx-PBtOx-1 polyplexes may contribute to differences in intracellular DNA availability, potentially influencing the relative transfection outcomes observed among the investigated systems. All copolymers exhibited high biocompatibility toward both cancerous H1299 and non-cancerous MRC-5 cell lines. Nevertheless, polymer structure and composition were found to influence the cytocompatibility of the amino-functionalized PEtOx-PBtOx copolymers and their corresponding polyplexes. In particular, the low TCP of PEtOx-PBtOx-2 under physiological conditions promotes mesoglobule formation, which has been associated with reduced polymer-membrane interactions and resulted in markedly high IC_{50} values exceeding $5 \text{ mg} \cdot \text{mL}^{-1}$. In contrast, involvement of primary amino groups in intra-molecular hydrogen bonding within PEtOx-PBtOx-1 may limit effective charge exposure, yielding cytocompatibility comparable to that of PEtOx-PBtOx-3. Altogether, the obtained results provide mechanistic insight into how polymer structure, amino functionality, and environmental responsiveness collectively govern the physicochemical behavior and biological responses of PEtOx-PBtOx-based vector systems. The study highlights the multifactorial nature of structure-property-behavior relationships in polymer-based non-viral gene delivery systems, which cannot be attributed

solely to the type of amino functionality but are inherently system-specific, and establishes a solid platform for further optimization and development.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym18040536/s1>, Figure S1: SEC traces of the polymeric precursor composed of EtOx and BtOx and EtOx-PBtOx copolymers functionalized with amino groups; Figure S2: Transmittance vs. temperature curves of amino-functionalized PEtOx-PBtOx copolymers; Figure S3: Representative Cryo-TEM micrographs and size distribution histograms of polyplexes prepared from PEtOx-PBtOx copolymers at N/P ratio of 8; Figure S4: Variations in EtBr fluorescence intensity after a competitive DNA displacement by dextran sulfate (DS); Figure S5: The cytotoxic activity of PEtOx-PBtOx copolymers and their corresponding polyplexes investigated in cancerous H1299 and the non-cancerous MRC5 cell lines.

Author Contributions: Conceptualization, N.O.-T. and E.H.; methodology, D.H., N.O.-T., M.P., I.U. and E.H.; software, D.H., N.O.-T., M.P., I.U. and E.H.; validation, N.O.-T., M.P., A.K., I.U., S.R. and E.H.; formal analysis, D.H., N.O.-T., M.P., A.K., I.U. and E.H.; investigation, D.H., N.O.-T., M.P., I.U. and E.H.; resources, N.O.-T., I.U., S.R. and E.H.; data curation, N.O.-T. and E.H.; writing—original draft preparation, D.H., N.O.-T., M.P., I.U. and E.H.; writing—review and editing, N.O.-T., M.P., A.K., I.U., S.R. and E.H.; visualization, D.H., N.O.-T., M.P. and E.H.; supervision, N.O.-T., A.K., I.U., S.R. and E.H.; project administration, N.O.-T. and E.H.; funding acquisition, N.O.-T., S.R. and E.H. All authors have read and agreed to the published version of the manuscript.

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