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Enhancing the Biocompatibility and Biodegradability of Linear Poly(ethylene imine) through Controlled Oxidation

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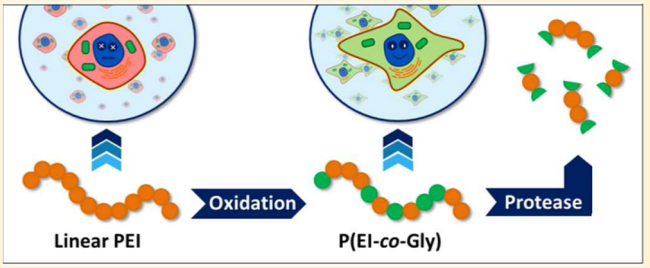
Supporting Information

ABSTRACT: (Bio)degradable poly(ethylene imine-*co*-glycine)s (P(EI-*co*-Gly)) were synthesized through efficient and controlled oxidation of linear poly(ethylene imine) (LPEI) using hydrogen peroxide in a methanol/water mixture. Temperature, peroxide concentration, and reaction time were varied to adjust the degree of oxidation (DO). At low temperatures, the oxidation process was found to be well-controlled with almost 85% of ethylene imine units converted to the corresponding amide. Importantly, oxidation of more than 10% of the LPEI rendered the polymers water-soluble. The oxidation reaction and molecular structure of P(EI-*co*-Gly)s were studied in detail by various nuclear magnetic resonance (NMR) methods, infrared (IR) spectroscopy, and size exclusion chromatography (SEC). The introduction of backbone amide groups to the P(EI-*co*-Gly)s facilitated its (bio)degradation under acidic conditions or by proteases. Moreover, the P(EI-*co*-Gly)s exhibited negligible cytotoxicity, particularly relative to LPEI. The interaction of the copolymers with serum-containing medium was investigated showing no indication of coagulation. Preliminary studies indicate that P(EI-*co*-Gly) is a promising biodegradable polymer with negligible toxicity in human cell lines.



INTRODUCTION

Poly(ethylene imine) (PEI) is one of the most successfully and widely studied vectors in nonviral gene delivery and is considered the gold standard for gene transfection.[1,2] The repeating ethylene amine structure of PEI can electrostatically interact with the DNA/RNA phosphate groups and form so-called polyplexes.[3−5] The polymer exists in branched or linear architecture, which influences the transfection behavior.[6,7] However, PEI suffers from several drawbacks such as cytotoxicity,[8−10] low hemocompatibility,[11] and nonbiodegradability,[12,13] which limits its clinical applications.

In the past decade, the chemical modification of PEI has garnered tremendous interest, with the primary objective of reducing cytotoxicity, increasing transfection efficiency and cell selectivity.[2,14−16] Most biological studies have focused on partially modified PEI conjugates.[17−19] Procedures have involved chemical transformation of the amine groups of PEI via alkylation, ring-opening of epoxides, and Michael addition, among others.[2]

Previously, MacDonald and co-workers reported the oxidation of branched PEI by hydrogen peroxide.[20] The authors demonstrated that the modification of the PEI backbone proceeds via the following intermediate species: hydroxylamine (**2**),[21] nitrone (**3**),[22,23] and oxaziridine (**4**)[23−25] (Scheme 1). The ring-opening of the oxaziridine, as postulated by MacDonald, leads to a Beckmann-type rearrangement product[26] and, subsequently, to the formation of the thermodynamic product, a stable amide group (**5**). The resulting structure resembles the repeating unit of poly(glycine) (PGly), which renders the polymers potentially biocompatible and degradable. However, this report claimed the oxidation to be accompanied by partial chain degradation, mainly referring to the appearance of carboxylic acid and aldehyde signals. These signals can, among others, occur from the oxidation (and subsequent hydrolysis) of primary amines present in branched PEI.[20] To this end, we utilize linear PEI for the presented study to prevent the influence of primary amines. Furthermore, the use of a linear polymer precursor simplifies the characterization due to the absence of primary and tertiary amines. Using

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Scheme 1. Schematic Representation of the Oxidation Mechanism of a Secondary Amine (1) via Hydroxylamine (2), Nitrone (3), and Oxaziridine (4) Intermediates To Form the Final Amide Group (5), As Proposed by MacDonald et al.[20]

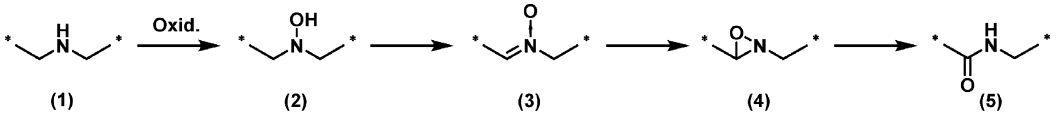
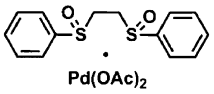
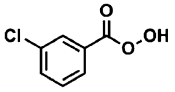


Table 1. Different Approaches for the Oxidation of Linear Poly(ethylene imine)

Oxidizing agent	Structure	Equivalents per EI unit	Solvent	Temperature [°C]	Time [h]	Degree of oxidation [%] (^{1}H NMR)
Oxone[29]	$KHSO_5$/ $KHSO_4$/ K_2SO_4	1.1	MeOH	18	16	degradation
White catalyst[30], O_2		0.1	MeOH	45	7	no oxidation
MCPBA		1.0	$CHCl_3$	18	16	no oxidation
Aq. H_2O_2		4.0	MeOH	18	16	80

modern characterization techniques and careful kinetic studies, we show that polymer degradation is minimal.

Surprisingly, since the initial report of the PEI oxidation, few (if any) similar reports have appeared in the past 40 years. The controlled oxidation of PEI represents a highly useful method for the modification of PEI and offers a unique opportunity to facilitate biocompatibility and degradability of an otherwise toxic PEI. Such an improvement would enable, for the first time, *in vivo* studies especially for applicability in gene delivery. Recently, Hauser and co-workers demonstrated that the oxidation of an already formed PEI/DNA polyplex would decrease the amount of cationic charges.[27] However, this contribution focused on the biological performance of these altered polyplexes and lacked details on the chemical nature of the modification.

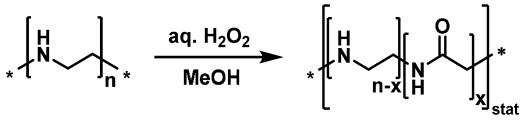
The goal of the present work was to understand the oxidation of PEI, to identify the optimal reagents/conditions to gain control over the oxidation process, and to evaluate the structure of the resulting polymers in detail. Therefore, we investigated the influence of several parameters, including time, temperature, and concentration, as well as the nature of the oxidizing agent in order to identify optimized conditions. A series of four P(EI-*co*-Gly) copolymers were synthesized and extensively analyzed. The degradation of the polymers was examined using HCl or trypsin and underlined the potential of the presented macromolecular system. Furthermore, serum stability and cytotoxicity were evaluated and found to be superior as compared to unmodified PEI.

RESULTS AND DISCUSSION

Oxidation of Linear Poly(ethylene imine). The first parameter investigated in the oxidation process of LPEI[28] was the oxidizing agent. Initial experiments focused on evaluating the suitability of various oxidizing agents (Table 1), where the degree of oxidation (DO) was assessed by ^{1}H NMR spectroscopy. Interestingly, the use of common oxidizing agents such as oxone, *m*-chloroperoxybenzoic acid (*m*CPBA), or the White catalyst (a palladium coordination complex) led to no measurable DO and, in some cases, degradation of the polymer. The oxidation of LPEI with hydrogen peroxide, however, led to oxidized species with a DO of up to 80%. The modified polymers exhibited an extraordinarily high water solubility for DOs > 10%. The simplicity of the oxidation reaction together with the ease of purification (precipitation in diethyl ether followed by freeze-drying) makes this procedure straightforward. For these reasons, hydrogen peroxide oxidation was the method used for all following investigations.

In the mechanism described above (Scheme 1), the oxidation of LPEI converts the latter into a partially oxidized poly(ethylene imine-*co*-glycine) (P(EI-*co*-Gly)) (Scheme 2), which

Scheme 2. Schematic Representation of the Oxidation of Commercial LPEI to P(EI-*co*-Gly) by Hydrogen Peroxide



consists of unaffected ethylene imine (EI) and newly formed glycine (Gly) units. Because of incomplete hydrolysis during its industrial synthesis process, the starting LPEI contains up to 5% 2-ethyl-2-oxazoline units.[31] These groups remain unaffected during the oxidation, which is shown by oxidation experiments of poly(2-ethyl-2-oxazoline) homopolymer (Supporting Information, Figure S1). Furthermore, the unaffected 2-ethyl-2-oxazoline (EtOx) repeating units were also used to quantify the DO. Since the signals of EI and Gly repeating units, respectively, are not entirely baseline separated, the error of the quantification of the DO is estimated as ±5%. The sharp signal at ~8.30 ppm could be attributed to degraded species or

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solvent impurities. However, the integral of the peak is lower than 2% as compared to the polymer signals.

The successful oxidation is exemplified by ^{1}H NMR analysis in Figure 1, which shows an overlay of LPEI and P(EI-*co*-Gly$_{67\%}$) spectra. As verified by HSQC NMR measurements (Figure S2), the newly formed signal F can be primarily ascribed to the CH_2 group of the glycine backbone. Signal E indicates the formation of an amide group showing no coupling to polymer associated carbon signals (see Figure S2).[32] Its appearance is rather surprising and cannot be used for quantification of the degree of oxidation (DO) due to proton exchange processes in D_2O. The ^{13}C NMR signal at ~170 ppm corresponds to the amide carbonyl group (no coupling was observed by heteronuclear NMR, HSQC). The DO was calculated from the integration of the ^{1}H NMR signals of the copolymer backbones via eq 1 (Experimental Section).

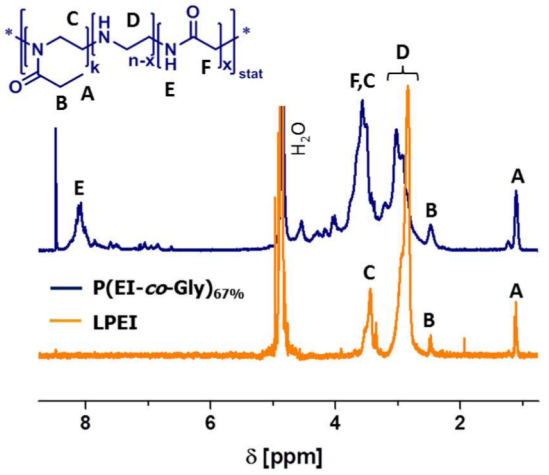

Figure 1. Comparison of ^{1}H NMR spectra of LPEI and P(EI-*co*-Gly) (including unaffected EtOx units, <5%) with a degree of oxidation of about 67% (D_2O, 400 MHz).

Kinetic Investigations. Synthetic control over the oxidation process is critical, so the oxidation reaction was investigated as a function of the concentration of peroxide, the temperature, and reaction time. First, the oxidation was performed with varying concentrations of hydrogen peroxide (0−1 equiv of H_2O_2 per EI repeating unit) for 20 h at 18 °C, the optimized time/temperature to observe a maximum DO (Figure S3). The increase in peroxide concentration resulted in a nearly linear increase of the DOs and represents a simple and effective method to control the DO. Moreover, with 0.9 equiv of H_2O_2 per EI unit, the DO reaches a maximum of 60%, whereas an excess of H_2O_2 (4 equiv) enables the oxidation of almost 85% of EI units. For this reason, excess of H_2O_2 was used for the following kinetic studies.

Starting from identical mixtures of LPEI, methanol, and H_2O_2, the oxidation reaction was performed at various temperatures and reaction times. The determined DOs from temperature- and time-dependent investigations (by ^{1}H NMR) resulted in the kinetic curves depicted in Figure 2. Increasing temperature led to faster oxidation and, hence, higher DOs. The short recording times of the curve associated with the reaction conducted at 0 °C is due to technical limitations, as it was difficult to maintain this temperature for longer time periods. Higher temperatures were also explored; a similar experiment at 55 °C resulted in a multitude of sharp signals in the ^{1}H NMR spectrum of the products, which are not typical for polymers with broad molar mass distributions, suggesting degradation to low molar mass species (Figure S4). We therefore concluded that ambient temperature (18 °C in our case) was sufficient for attaining high DOs of up to 85% without causing polymer degradation. In summary, adjusting the H_2O_2 concentration, time, and temperature represents a simple and effective method to control the DO.

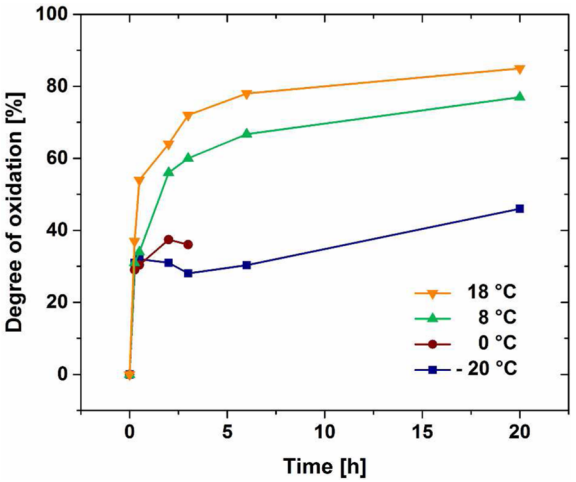

Figure 2. Kinetic studies of the oxidation of LPEI at different temperatures with a 4-fold excess of H_2O_2. Degrees of oxidation were calculated from ^{1}H NMR spectra (D_2O, 400 MHz).

P(EI-*co*-Gly) with Varying DOs. The ability to adjust the DO via temperature and time enabled the synthesis of a series of P(EI-*co*-Gly)s with varying amounts of glycine units (Table 2, **1**−**4**). To avoid the investigation of potential degradation products, centrifugal concentration was performed for all P(EI-*co*-Gly) samples using disposable Vivaspin 20 ultrafiltration devices with a molar mass cutoff (MWCO) of 3000 g mol^{-1}.

Larger H_2O_2 concentrations and higher temperatures resulted in a faster and less controllable oxidation. Therefore, to achieve polymers with lower DOs (e.g., **1** and **2**), the reaction was performed at 0 °C. Higher oxidation degrees (**3** and **4**) were obtained at 18 °C followed by purification through precipitation and freeze-drying.

An overlay of the ^{1}H NMR spectra of **1**−**4** can be found in Figure 3. A downfield shift of the LPEI backbone signal D to signal F as well as the appearance of the amide signal around 7.90 ppm (E) indicates a successful oxidation.

Diffusion-ordered NMR spectroscopy (DOSY NMR) confirms the presence of a single (polymeric) species, as indicated by the diffusion coefficient and, hence, the absence of degradation (Figure S5). Diffusion coefficients are specified in Table 2. Missing Mark−Houwink values, α and K, for the copolymers hinder the calculation of the molar weights via the Mark−Houwink equation. For LPEI, the molar mass (M_n) was determined as 4200 g mol^{-1} by viscosity measurements using literature values.[33] The viscosity of methanol (determined by viscosity measurements using a capillary/ball combination) and the diffusion coefficients were used to calculate the hydrodynamic radii R_0 of the (co)polymers via the Einstein−Smoluchowski relation (Table 2). Because of equipment dependent data, the diffusion coefficient from the residual water peak was used to "standardize" R_0 values of the (co)polymers by simple correction to literature reported value of D_{H_2O}.

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Table 2. Parameters for P(EI-*co*-Gly)s 1−4 and Starting LPEI at 25 °C: Degree of Oxidation (DO), Number-Averaged Molar Mass M_n (NMR, SEC), Diffusion Coefficient D (DOSY), Intrinsic Viscosity μ_{int}, and Hydrodynamic Radius R_0 According to the Einstein−Smoluchowski Relation

			M_n [g mol^{-1}]				
no.	composition	DO[a] [%]	NMR[b]	SEC (DMAc)[c,g]	D[d] [10^{-10} m^2 s^{-1}]	μ_{int}[e] [mL g^{-1}]	R_0[f] [nm]
	LPEI		6500[36]	1100	2.8	28.6	3.07
1	P(EI_{80}-*co*-Gly_{20})	20	6900	1500	6.3	6.9	1.37
2	P(EI_{60}-*co*-Gly_{40})	40	7300	1400	8.9	8.0	0.97
3	P(EI_{33}-*co*-Gly_{67})	67	7900	1700	6.8	8.6	1.27
4	P(EI_{15}-*co*-Gly_{85})	85	8200	1600	7.9	8.3	1.09

[a]Percentage of formed GLY units determined by ^{1}H NMR spectroscopy. [b]Determined by ^{1}H NMR (calculated from LPEI: 6500 g mol^{-1},[36] ratio of PEI and GLY). [c]Determined by SEC (eluent: DMAc + 0.21% LiCl, calibration against polystyrene standard). [d]Determined by diffusion-ordered NMR spectroscopy (DOSY, D_2O, 400 MHz, 25 °C, see Figure S4). [e]Determined by viscosity measurements (MeOH, 25 °C). [f]Calculated by Einstein−Smoluchowski relation by means of $\eta_{0,MeOH}$ = 0.61 × 10^{-3} kg m^{-1} s^{-1} (Experimental Section, eq 2).[37] The diffusion coefficient from residual water peak (D_{H_2O} = 5.60 × 10^{-9} m^2 s^{-1} (25 °C)) was used to "standardize" R_0 values by simple correction to literature reported value of $D_{H_2O,Lit}$ = 2.30 × 10^{-9} m^2 s^{-1} (25 °C).[38] [g]All samples are hardly soluble in the SEC eluent DMAc. Unfortunately, there is no calibration standard available for cationic polymers like LPEI and derivatives.

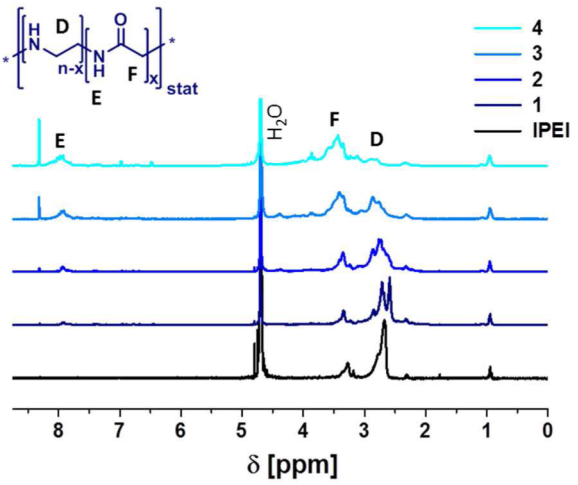

Figure 3. ^{1}H NMR spectra of LPEI and P(EI-*co*-Gly)s **1−4** (D_2O, 400 MHz).

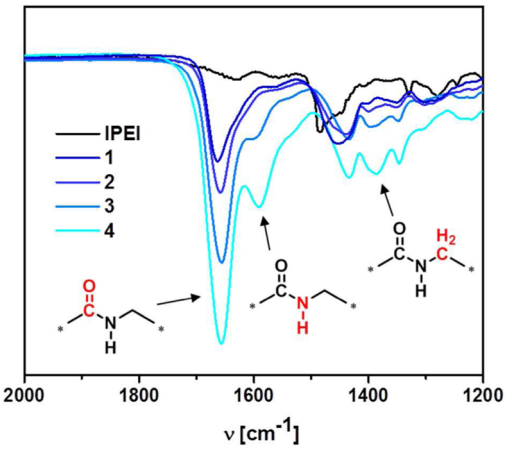

Figure 4. IR spectra of P(EI-*co*-Gly)s **1−4** and LPEI in the wavenumber range from 1200 to 2000 cm^{-1}.

A difference in R_0 of LPEI and P(EI-*co*-Gly)s can be explained by two factors: first, hydrogen bonding, which occurs due to the presence of the newly formed amide groups, and second, decreasing charges owing to the disappearance of secondary amines. This allows an entanglement of the polymer chains, and as a result, smaller hydrodynamic radii are observed for the oxidized species. However, the larger R_0 of LPEI could also be attributed to the formation of aggregates as the polymer is known to form hydrogen bonds in aqueous solution.[34] Unfortunately, asymmetric flow field flow fractionation (AF4, RC membrane with MWCO of 10 000 g mol^{-1}), a well-established method for the detailed characterization of cationic polymers,[35] was not successful due to the low molar masses of the starting LPEI and the corresponding oxidized copolymers (<10 000 g mol^{-1}).

The IR spectra (in the wavenumber region between 1200 and 2000 cm^{-1}) of LPEI and **1−4** are shown in Figure 4. Numerous investigations regarding IR spectroscopy of polyglycine are known from the literature.[39−41] The vibration at 1660 cm^{-1} results from the carbonyl stretching vibration of the secondary amide group in P(EI-*co*-Gly).[20,42] This signifies a successful oxidation. Signals attributable to carboxylic acid derivatives, as possible degradation products, are expected to appear at higher wavenumbers (>1700 cm^{-1});[43] however, they are not observed. Starting from LPEI (no carbonyl vibration), increased DOs lead to more intense carbonyl bands. The signal at 1600 cm^{-1} is likewise assigned to amide mode. The vibrations between 1400 and 1340 cm^{-1} can be assigned to the CH_2 bending and wagging mode of the formed glycine, respectively. The 1480 cm^{-1} band of the LPEI-CH_2 backbone gradually decreases in wavenumber (to 1435 cm^{-1}) with an increase of DO.

Poor solubility in common solvents and expected interactions of the cationic polymer with the column material in the case of aqueous SEC setups prohibit successful determination of molar masses of the P(EI-*co*-Gly) by size exclusion measurements. However, LPEI and copolymers **1−4** could be dissolved in *N*,*N*-dimethylacetamide (DMAc) at low concentrations. The appearance of the LPEI signal at higher elution volumes, and hence at lower molar masses, compared to the P(EI-*co*-Gly)s is another indication for the absence of quantitative degradation during oxidation (Figure S6). Unfortunately, a calibration for cationic polymers is not available, which complicates the determination of accurate molar masses by SEC.

Degradation Studies. One potential advantage of P(EI-*co*-Gly) compared to unmodified LPEI is the possibility of biodegradation due to the incorporation of amide bonds in the

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polymer backbone. To investigate the decomposition behavior, P(EI-*co*-Gly$_{67\%}$) (**3**) was chosen as a model polymer and subjected to acidic (6 mol L^{-1} HCl) and enzymatic (trypsin) conditions. While the pure LPEI showed no changes under these conditions, the ^{1}H NMR spectra of the oxidized species revealed sharp and undefined signals (comparable to the oxidation reactions at high temperatures), usually atypical for polymers with broad molar mass distributions and chain lengths of around 150 units (Figure 5). Further evidence of a

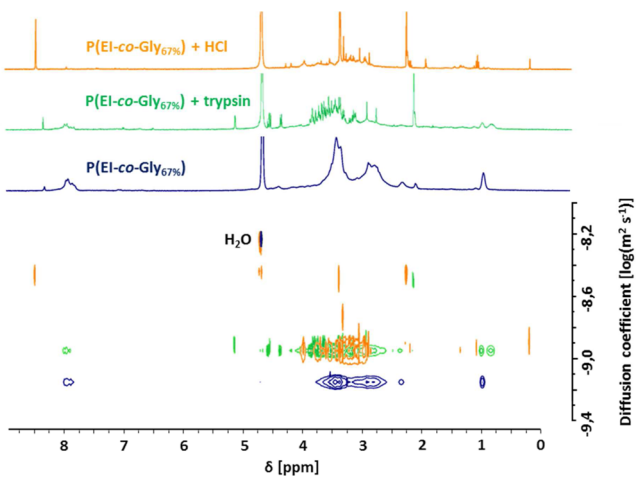

Figure 5. DOSY NMR of **3** before (blue) and after treatment with 6 mol L^{-1} aqueous hydrogen chloride (orange) and peptidase (trypsin) (green) (D_2O, 400 MHz, 25 °C).

successful degradation is given by the splitting of the signals of the EtOx side chain and the reduction in the peak intensity of the amide bond at 7.9 ppm, which completely disappeared upon the treatment with HCl. Simultaneously, the intensity of the sharp signal at 8.45 ppm increases, validating the earlier assumption of this signal being attributed to degradation products. Furthermore, successful degradation was confirmed utilizing DOSY NMR (Figure 5). Polymer degradation results in increased diffusion coefficients as well as a fractionation of the signals. The signal of water was used for calibration.

Size exclusion chromatography in DMAc confirmed the successful degradation (Figure S7). The improved solubility and the shift to lower molar masses further support our claim.

Cytotoxicity and Serum Stability. Another important parameter for a candidate polymer for biomedical applications is its biocompatibility. Pure PEI, although known as a gold standard for nonviral gene delivery,[1,2] possesses a high cytotoxicity due to its high cationic charge density. Since oxidation of LPEI results in repeating units similar to natural occurring peptides, we hypothesized that the cytocompatibility of oxidized LPEI (i.e., P(EI-*co*-Gly)) should exceed that of PEI. Such an improvement would enable *in vivo* studies especially for gene delivery applications. In order to evaluate this parameter, cytotoxicity was investigated by 3-[4,5-dimenthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay using human embryonic kidney 293 cell line (HEK 293) (Figure 6A).

While LPEI induces a toxic effect already at very low concentrations (<6.25 mg L^{-1}), all copolymers investigated are cytocompatible up to a concentration of 25 mg L^{-1}. Furthermore, a noticeable effect of the DO on the cell viability can be observed. For example, while cell viability values drop below 60% after incubation with P(EI-*co*-Gly$_{20\%}$) (**1**) at 50 mg L^{-1} for 48 h, P(EI-*co*-Gly$_{85\%}$) (**4**) still does not induce harmful effects, with ∼80% cell viability even at relatively high concentrations (200 mg L^{-1}).

A second important parameter, the interactions of polymers with serum proteins, was assessed by DLS measurements of P(EI-*co*-Gly)s after treatment with fetal bovine serum (FBS) (Figure 6B). All investigated copolymers resulted in *Z*-average values that were indistinguishable from the untreated control sample (pure FBS), indicating no further aggregation and, hence, polymer stability in the presence of serum proteins over 48 h. LPEI itself could not be investigated since its addition to serum resulted in an immediate precipitation of protein components. It should be noted that this investigation does only demonstrate the absence of serum coagulation, while an interaction of protein and polymer is still possible. In conclusion, it can be stated that even a low DO is able to shield the copolymer from interacting with cell membrane/proteins and therefore significantly reduces harmful effects on cells, as shown by the cytotoxicity investigations.

EXPERIMENTAL SECTION

Materials. Reagents were used as received unless otherwise noted. LPEI (MW 25 000 g mol^{-1}) was obtained from Polysciences. Aqueous hydrogen peroxide solution (30% w/w) and methanol were purchased from Sigma-Aldrich (USA). Poly(2-ethyl-2-oxazoline) with a degree of polymerization of 95 was synthesized according to the literature starting from 2-ethyl-2-oxazoline (Sigma-Aldrich).[44] Hydrochloric acid and trypsin were purchased from Sigma-Aldrich. Centrifugal

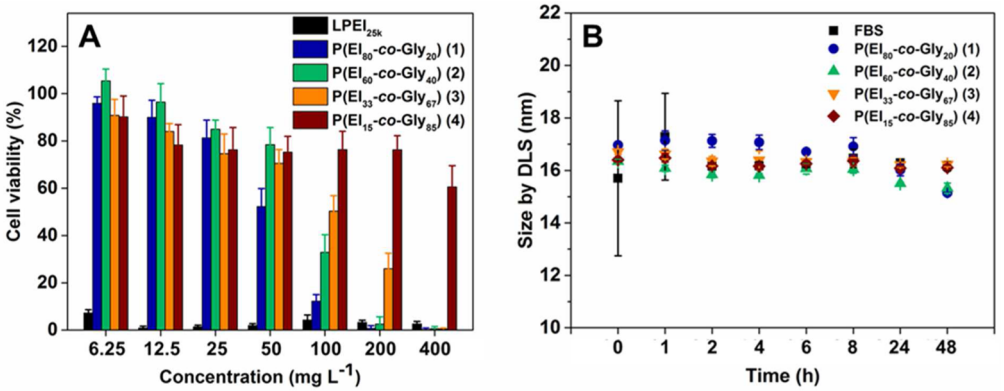

Figure 6. (A) Cytotoxicity of LPEI as well as P(EI-*co*-Gly)s **1**−**4** by MTT assay on HEK 293 cells. (B) Serum stability by time-dependent DLS measurements of FBS in the presence of P(EI-*co*-Gly)s **1**−**4**.

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concentration was performed using disposable Vivaspin 20 ultrafiltration devices with a molar mass cutoff (MWCO) of 3000 g mol^{-1}.

General Methods and Instrumentation. Proton (^{1}H) nuclear magnetic resonance (NMR) and carbon (^{13}C) NMR spectra were acquired in D_2O, at room temperature using a Bruker AC 400 MHz or a Bruker AC 300 MHz (100 MHz ^{13}C); chemical shifts (δ) are expressed in parts per million relative to TMS. ^{1}H NMR measurements were carried out with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208 Hz spectral width, and 32K data points.

The degree of oxidation (DO) was calculated from the integrals of the ^{1}H NMR signals of the copolymer backbones (see Figure 1 and Figure S2) using eq 1

$$\mathrm{DO} = \frac{2\left(F - \frac{4}{3}A\right)}{2\left(F - \frac{4}{3}A\right) + D} \quad (1)$$

where F is the integral of NH−CO−CH_2, A is the integral of CH_2−CH_3 (provided that $A \ll F$), and D is the integral of NH−CH_2−CH_2. Diffusion-ordered NMR spectroscopy (DOSY) was performed on a Bruker 400 MHz Avance I NMR system equipped with a BBO z-gradient probehead. 2D spectra were recorded using a pulse programm (Bruker: "ledbpgp2s") with bipolar gradient pulses and two spoil gradients.[45] Experimental parameters were optimized once using a 1D version of the aforementioned pulse sequence and then kept constant for all samples: 32 number of scans, recycle delay of 2 s, acquisition time of 1.7 s, diffusion time ($d20$) of 75 ms, a gradient pulse length ($p30$) of 2.5 ms, and a linear gradient amplitude ramp ranging from 5 to 95% (with respect to the maximum strength of 53.5 G/cm) with 64 increments. Sample temperature was set to 25 °C. Standard DOSY processing algorithms provides by TopSpin were utilized to obtain the final DOSY spectra and to extract the diffusion coefficients.

Size exclusion chromatographies (SEC) were performed on an Agilent Technologies 1200 Series gel permeation chromatography system equipped with a G1329A autosampler, a G131A isocratic pump, a G1362A refractive index detector, and both a PSS Gram 30 and a PSS Gram 1000 column placed in series. As eluent, a 0.21% LiCl solution in *N*,*N*-dimethylacetamide (DMAc) was used at 1 mL min^{-1} flow rate and a column oven temperature of 40 °C. Molar masses were calculated using poly(styrene) standard.

Fourier transform infrared (FT-IR) spectra were recorded on a Thermo Nicolet Nexus 670 FT-IR spectrometer, using the attenuated total reflectance (ATR) method.

Viscosity measurements were conducted using an AMVn viscometer (Anton Paar, Graz, Austria), with the capillary/ball combination of the measuring system. The respective flow times for the solvent (methanol) and polymer solutions, τ_0 and t, were measured at 25 °C, with relative viscosities $\eta_r = t/\tau_0$ in the range of 1.1−1.8, which corresponds to dilute solutions.

The hydrodynamic radius R_0 was calculated from diffusion coefficients D (DOSY) and liquids viscosity η_0 by eq 2, the Einstein−Smoluchowski relation.

$$R_0 = \frac{k_B T}{6\pi\eta_0 D} \quad (2)$$

where k_B is the Boltzmann constant, T is the temperature, η_0 is the liquid's viscosity, and D is the diffusion coefficient.

Oxidation of Linear Poly(ethylene imine). The synthesis is exemplified by the P(EI-*co*-$Gly_{67\%}$) with a degree of oxidation (DO) about 67%. LPEI (0.025 g) was dissolved in methanol (V = 0.63 mL). After stirring for 5 min, aqueous hydrogen peroxide solution (4 equiv per EI unit, 0.125 mL, 30% w/w) was added dropwise into the stirring polymer mixture at room temperature (water bath, 18 °C). After 2 h, the mixture was precipitated two times into 15 mL of ice-cold diethyl ether. The oxidized polymer was filtered off and washed with 5 mL of diethyl ether. The residue was dissolved in 3 mL distilled water and freeze-dried overnight (yield: 95%).

LPEI. ^{1}H NMR (400 MHz, D_2O): δ 3.46−3.28 (NH−CH_2−CH_2), 3.00−2.60 (NH−CH_2), 2.35 (CH_2 EtOx), 0.99 (CH_3 EtOx) ppm. ^{13}C NMR (100 MHz, D_2O): δ 148.5 (CO−CH_2−CH_3), 47.1 (NH−CH_2−CH_2), 25.1 (CH_2−CH_3), 9.8 (CH_2−CH_3) ppm. FT-IR: $\tilde{\nu}$ 3180 (OH, NH), 2870 (CH asym/sym str), 1480 (C−H def), 1280, 1125 (C−N str), 1045 (C−N) cm^{-1}.

P(EI-co-GLY$_{67\%}$). DO = 67%, μ_{Int} = 8.6 mL g^{-1}. ^{1}H NMR (400 MHz, D_2O): δ 8.30, 8.05−7.75 (N*H*−CO−CH_2), 3.75−3.18 (NH−CO−CH_2), 3.14−2.41 (NH−CH_2−CH_2), 2.30 (CH_2−CH_3), 1.94 (CH_2−CH_3) ppm. ^{13}C NMR (100 MHz, D_2O): δ 171.1 (NH−*C*O−CH_2), 166.5, 140.5 (*C*O−CH_2−CH_3), 47.0−34.7 (NH−*C*H_2−*C*H_2, NH−CO−*C*H_2), 25.2 (CH_2−CH_3), 8.5 (CH_2−CH_3) ppm. FT-IR: $\tilde{\nu}$ 3260 (OH, NH), 2870 (CH asym/sym str), 1658 (C═O), 1600 (NH bending), 1435 (C−H def), 1395, 1340 ($CH_{2,Gly}$ asym/sym str), 1155 (C−N str), 1065 (C−N) cm^{-1}.

Kinetic Investigations. The peroxide concentration dependent oxidation of linear poly(ethylene imine) was performed according to the general procedure described above. For further kinetic investigations, the H_2O_2 concentration was set constant to 4 equiv per EI unit to reach a maximum level of oxidation. To investigate the influence of temperature, five analogue stock solutions of LPEI, ethanol, and H_2O_2 were prepared and stirred at selected temperatures (ranging from −20 to 55 °C). After defined time ranges (ranging from 0.5 to 20 h) aliquots were taken and purified by precipitation. After washing with diethyl ether the samples were freeze-dried. The degree of oxidation was determined by proton nuclear magnetic resonance measurements (^{1}H NMR).

P(EI-*co*-Gly) with Varying DOs. The oxidation of linear poly(ethylene imine) was performed in larger scales according to the general procedure described above. Since larger amounts of H_2O_2 lead to higher generation of heat and, hence, to a faster and more uncontrollable oxidation, the reaction for lower oxidation samples **1** and **2** was performed at 0 °C. Higher oxidation degrees (**3** and **4**) were obtained at room temperature (18 °C).

Degradation Studies. Polymer **1** (DO 67%, 20 mg) was dissolved in 6 mol L^{-1} HCl (2 mL) and stirred for 20 h at 70 °C. Subsequently, the mixture was neutralized with sodium hydroxide and freeze-dried. A second approach was the addition of the protease Trypsin (0.5 mL) to an aqueous solution of **1** (5 mg in 1 mL of H_2O). After stirring for 20 h at 37 °C, the mixture was freeze-dried. Both products were analyzed by ^{1}H and DOSY NMR and SEC.

Cytotoxicity. HEK 293 cells were cultured in RPMI-1640 supplied with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. HEK 293 cells were seeded onto 96-well plates at a density of 10 000 cells/well. The cells were incubated at 37 °C, 5% CO_2. After 24 h, the medium was replaced with fresh medium containing polymer at various concentrations. After incubation for 48 h, 100 μL of fresh medium and 20 μL of 5 mg/mL MTT solution were used to replace the sample medium. After 4 h of incubation, the medium was removed, and DMSO (150 μL) was added to each well to dissolve the formazan crystals. The absorbance of each well was measured with a microplate reader (Power-Wave X, Biotek Instruments, USA) at 690 nm as reference and subtracted from the absorbance of the same well at 550 nm. The results were presented as a percentage of absorbance of the blank control.

Serum Stability. Polymers were dissolved in PBS containing 10% FBS. The particle sizes within the polymer solutions were analyzed using a Zetasizer 3000 HAS (Malvern Instrument Ltd., Malvern, UK) equipped with a He−Ne laser beam at 658 nm (scattering angle: 90°) over 48 h. The concentration of the polymers was 500 mg/L. Each sample was measured three times, and an average particle size was obtained.

CONCLUSION

A method for the tailored oxidation of commercially available linear poly(ethylene imine) (LPEI) was developed. The 1972 report by MacDonald et al. on the oxidation of branched poly(ethylene imine) was revisited by means of extensive kinetic studies and characterizations of the modified LPEIs (NMR, DOSY, IR, SEC, viscometry). Concentration as well as temperature proved efficient parameters to control the degree

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of oxidation of poly(ethylene imine-*co*-glycine)s (P(EI-*co*-Gly)) copolymers. At low temperatures, the oxidation process was found to be well-controlled, and a maximum of 85% oxidized EI units could be reached. Notably, oxidation of only 10% of the LPEI led to water-soluble polymers. The formed amide groups render the copolymers (bio)degradable, as shown by the decomposition experiments using aqueous hydrogen chloride and trypsin. Finally, the biocompatibility improvement of P(EI-*co*-Gly)s compared to LPEI was demonstrated by cytotoxicity investigations and serum stability measurements.

The controlled oxidation of LPEI, as shown in this contribution, enables the specific improvement of the (bio)-properties, such as biodegradation and biocompatibility, and hence helps to overcome the main drawbacks of the initial material. It can be assumed that there is an optimal composition of copolymers to reach high transfection efficiencies (EI content) while showing good cytocompatibility (Gly content). However, the investigation of the transfection abilities will be the focus of further studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.5b01940.

Figures S1−S7 (PDF)

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Notes

The authors declare no competing financial interest.

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