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Linear Poly(ethylene imine)-Based Hydrogels for Effective Binding and Release of DNA

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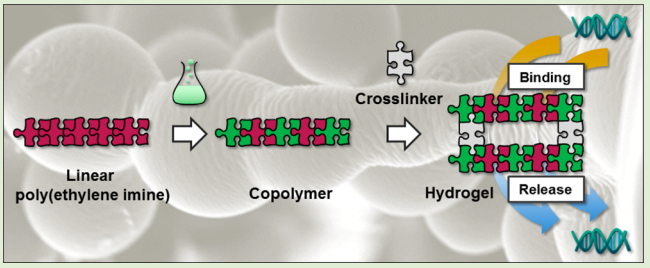
Supporting Information

ABSTRACT: A series of copolymers containing both amine groups of linear poly(ethylene imine) (LPEI) and double bonds of poly(2-(3-butenyl)-2-oxazoline) (PButEnOx) was prepared. To this end, a poly(2-ethyl-2-oxazoline) (PEtOx) precursor was hydrolyzed to the respective LPEI and functionalized in an amidation reaction with butenyl groups resulting in the double bond containing poly(2-(3-butenyl-2-oxazoline)-*co*-ethylene imine) (P(ButEnOx-*co*-EI)). Hydrogels were obtained by cross-linking with dithiols under UV-irradiation resulting in networks with different properties in dependence of the content of double bonds. The developed method allows the exact control of the amount of ethylene imine units within the copolymer and, thus, within the resulting hydrogels. The gel structures were characterized by solid state NMR and infrared spectroscopy. In addition the water uptake behavior from the liquid and the gas phase was investigated. It was shown by an ethidium bromide assay (EBA) that the copolymers and the respective hydrogels were able to bind and release DNA. Furthermore, the influence of the ethylene imine content on this interaction was investigated.



INTRODUCTION

The fast and efficient detection of pathogenes is of tremendous interest nowadays, ranging from applications in agriculture to medicine. Each species of pathogen carries a unique set of DNA and RNA sequences, which can be potentially detected by hybridization with another DNA strand containing complementary nucleic acid sequences. This approach is exploited by DNA biochips that consist of DNA sequences covalently bound/attached to solid substrates like glass,[1,2] silicon, gold,[3] or polymers such as poly(methyl methacrylate).[4] However, an essential prerequisite for a successful detection is the isolation and purification of nucleic acids from highly complex samples, such as blood and feces.[5] For this purpose, materials that can specifically and reversibly bind genetic materials are of significant interest. In this context, the interaction between DNA and cationic polymers has been investigated intensely. In particular, poly(ethylene imine) (PEI) has been widely studied, since its amine groups interact effectively with the DNA/RNA phosphate groups, forming a so-called polyplex.[6−11]

The major drawbacks of two-dimensional DNA chips are the limited loading capacity of surface materials and the restricted hybridization efficiency.[12,13] An alternative approach, overcoming these issues, is the reversible binding of genetic materials within a three-dimensional network. In this way a considerable increase of the loading capacity compared to a two-dimensional system can be achieved.[14,15] A special class of three-dimensional networks are the so-called hydrogels. Although insoluble in any solvents, they can incorporate water up to a multitude of their own mass. This property allows the encapsulation and rapid diffusion of DNA molecules inside the network. The immobilization of DNA and other biomolecules within hydrogel-like structures has been recently the topic of intensive research.[16−19]

In this contribution we focus on the synthesis of three-dimensional networks based on linear poly(ethylene imine) (LPEI). The most common method for the formation of PEI-based hydrogels is the cross-linking of the amine groups using difunctional compounds like diglycidyl ethers or diisocyanates.[20−26] However, due to the insolubility of the network, it is not possible to determine the amount of amine groups that remain after the cross-linking process. For the binding and release of genetic material, it is essential to know the exact amount of amine groups. Hence, it is desirable to perform the cross-linking without decreasing the amine binding sides. To this end, a second functionality needs to be incorporated into the hydrogel precursor, which can be exploited for cross-linking. Possible candidates could be partially hydrolyzed double bond bearing poly(2-oxazoline)-based homo- and copolymers.[27] However, the double bonds of poly(2-(3-butenyl)-2-oxazoline) (PButEnOx)[28−30] and poly(2-(9-decen-

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yl)-2-oxazoline) (PDecEnOx)[31−33] based polymers do not stand the harsh hydrolysis conditions required to cleave of the oxazoline side chains. To overcome this problem, the alkene functionalities have to be introduced subsequently to hydrolysis.

This report introduces a new route to obtain LPEI-hydrogels with an adjustable amount of amine groups. To this end, we have synthesized an eight-membered library of poly(2-(3-butenyl)-2-oxazoline-*co*-ethylene imine) (P(ButEnOx-*co*-EI) copolymers by reaction of LPEI with *N*-succinimidyl-4-pentenate. Subsequent cross-linking via thiol−ene photoaddition leads to the formation of LPEI-containing hydrogels with a predetermined amount of amine groups.

Both the copolymers and the hydrogels are characterized by means of NMR and IR spectroscopies. The synthesized systems are investigated regarding their structure and swelling ability. The DNA interaction of the copolymers and the hydrogels is studied using the ethidium bromide assay (EBA; DNA binding) and a heparin assay (DNA release).

EXPERIMENTAL SECTION

Materials. PEtOx (50,000 g/mol), pyridine, 4-*N*,*N*-dimethylaminopyridine (DMAP), ethanol, 3,6-dioxaoctane-1,8-dithiol, and 2,2-dimethoxy-2-phenylacetophenone (DMPA), and genomic DNA sodium salt from herring testes (gDNA) were obtained from Sigma Aldrich. Ethidium bromide solution (1%) was purchased from Carl Roth (Karlsruhe, Germany). *N*-Succinimidyl-4-pentenate was prepared according to literature procedures.[28]

General Methods and Instrumentation. The hydrolysis of PEtOx was performed under microwave irradiation in an Initiator Sixty single-mode microwave synthesizer from Biotage, equipped with a noninvasive IR sensor (accuracy 2%). ^{1}H NMR spectra were recorded at room temperature using a Bruker AC 300 MHz spectrometer or a Bruker AC 250 MHz spectrometer, respectively. ^{13}C NMR was recorded at 100 MHz. Chemical shifts are reported in parts per million (ppm, δ scale) relative to the residual signal of the deuterated methanol. Solid state NMR measurements were performed on a Bruker Avance 400 spectrometer. The dry hydrogels were measured with 4 mm zircon-oxid rotors at 12.5 kHz. Swollen samples (12 h in D_2O) were measured using Kel-F rotors from Bruker-Biospin at 6.5 kHz. Tetrakis-trimethylsilylsilan (^{1}H) and adamantan (^{13}C) were used as standards. ^{1}H NMR was measured for 6 s, for ^{13}C NMR 70000 scans were carried out. 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) as standard. Fourier transform infrared (FT-IR) spectra were recorded on a FT-IR spectrometer IRAffinity-1 (Shimadzu) using the reflection method.

The water uptake measurements from the gas phase were performed using a TGA Q5000 SA thermogravimetric analyzer from TA Instruments Eschborn, Germany. For the data evaluation the "Universal Analysis" software was used. The water uptake of the hydrogels from the liquid state was measured gravimetrically. The used filter tubes were centrifuged with a Multifuge 1S-R from Thermo Scientific, and the swelling values (Q_{eq}) were calculated according to literature.[34] The fluorescence measurements were recorded on the microplate reader Infinite M200 PRO. The samples were excited at 525 nm and the emission was measured at a wavelength of 605 nm in a temperature range between 25 and 30 °C. Well plates were obtained from Sigma-Aldrich (NunclonTM Surface, 96 well, F-bottom) and centrifuge filter tubes from Costar (Spin-X, cellulose acetate membrane, 0.45 μm).

Microscopic detection of the DNA binding of hydrogels was performed using a fluorescence microscope (Cell Observer Z1, Carl Zeiss, Jena, Germany) equipped with a mercury arc UV lamp and the appropriate filter combinations for excitation and detection of emission. The fluorescence signal was captured directly in the wells. Images of a series (11 × 11 pictures per well) were captured with a 10× objective using identical instrument settings (e.g., UV lamp power, integration time, and camera gain) and spots of the 12-well plate were addressed using an automated XY table.

Linear Poly(ethylene imine). Poly(2-ethyl-2-oxazoline) (50,000 g/mol, PDI = 2.0; 5.0 g) was treated with an excess of 6 M aqueous hydrochloric acid (30 mL) for 1 h at 130 °C in a microwave synthesizer.[35] The excess of HCl and the resulting propionic acid were removed under reduced pressure and the obtained LPEI hydrochloride was dissolved in water. After addition of 3 M NaOH up to a pH value of 9, precipitation occurred. The LPEI was filtered off, recrystallized from water, dissolved in methanol, and precipitated into 650 mL of ice-cold diethyl ether. The white solid was dried at 85 °C under high vacuum and the degree of hydrolysis was determined by ^{1}H NMR by correlating the integrals of the PEI backbone and the remaining methyl group of the PEtOx side chain (yield: 3.0 g). ^{1}H NMR (250 MHz, MeOD): δ 2.95−2.52 (N-CH_2), 1.45−0.81 (CH_3 EtOx) ppm. FT-IR (ATR): 3175 (OH, NH), 2866 (CH asym/sym str), 1485 (C−H def), 1281, 1134 (C−N str), 1045 (C−N) cm^{-1}.

***N*-Succinimidyl-4-pentenate.** The synthesis of *N*-succinimidyl-4-pentenate was performed with minor changes according to a procedure described earlier by Gress et al.[28] 4-Pentenoic acid (30.738 g, 307 mmol) and *N*-hydroxysuccinimide (56.401 g, 490 mmol) were dissolved in 800 mL of dichloromethane. To the solution, *N*,*N*′-dicyclcohexylcarbodiimide (74.334 g, 360 mmol) was added. After stirring for 23 h at room temperature, the white precipitate was filtered off and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 450 mL of diethyl ether and 150 mL of water. After separation, the organic phase was washed five times with water. The organic layer was dried over sodium sulfate and the solvent was removed. The high viscous product was dried in high vacuum (5 × 10^{-2} mbar) at room temperature for three days and crystallized in the freezer at −18 °C for two days. The activated acid, *N*-succinimidyl-4-pentenate, was obtained as a white solid (yield: 54.9 g, 91%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 5.82 (*H*C═CH_2), 5.03 (HC═CH_2), 2.82 (CH_2 NHS), 2.70 (CH_2-CH_2-CO), 2.48 (CH_2-CH_2-CO) ppm. FT-IR (ATR): 2932 (CH asym/sym str), 1802 (CH═CH), 1728 (C═O), 1643, 1528 (C−H def), 1369, 1204 (C−N str), 1065 (C−N str) cm^{-1}.

Poly(2-(3-butenyl)-2-oxazoline-*co*-ethylene imine). The synthesis is exemplified by the copolymer with a 50% PEI content. LPEI (0.475 g) and the catalyst 4-*N*,*N*-dimethylamino-pyridine (DMAP, 0.117 g, 1.0 mmol) were dissolved in a microwave vial in pyridine at 80 °C. In a second vial, a defined quantity of *N*-succinimidyl-4-pentenate (1 equiv per EI unit, 1.015 g, 5.2 mmol for 50% PEI) was dissolved in the same solvent. The two solutions were combined to a 4 wt % mixture of LPEI and stirred for 20 h at 80 °C. Afterward, the mixture was added dropwise into 500 mL of cold diethyl ether. The precipitated copolymer was filtered off and washed with 40 mL of diethyl ether. The residue was dried under reduced pressure to constant weight. The side product *N*-hydroxysuccinimide (NHS) was removed by dialysis in a regenerated cellulose membrane (Spectra/Por, Rancho Dominguez, CA, molar mass cutoff 3500 g/mol) against water. ^{1}H NMR (250 MHz, MeOD): δ 6.00−5.74 (*H*C═CH_2), 5.18−4.93 (HC═CH_2), 3.79−3.36 (NR-CH_2-CH_2), 3.12−2.61 (NH-CH_2-CH_2), 2.63−2.59 (NHS), 2.60−2.17 (CH_2 ButEnOx), 1.41−0.80 (CH_3 EtOx) ppm, PEI = 50%. SEC (DMAc, LiCl): M_n = 10700 g·mol^{-1}, PDI = 1.44. FT-IR (ATR): 3280 (OH, NH), 2870 (CH asym/sym str), 1640 (C═O), 1420 (C−H def), 1240 (C−N str), 1080 (C−N str) cm^{-1}.

Poly(2-(3-butenyl)-2-oxazoline-*co*-ethylene imine)-Based Hydrogel. The synthesis is exemplified by the gel starting from the 50% PEI containing copolymer described above. In a microwave vial, poly(2-(3-butenyl)-2-oxazoline-*co*-ethylene imine) with 50% PEI content (0.103 g) was dissolved in ethanol. In a second vial, the

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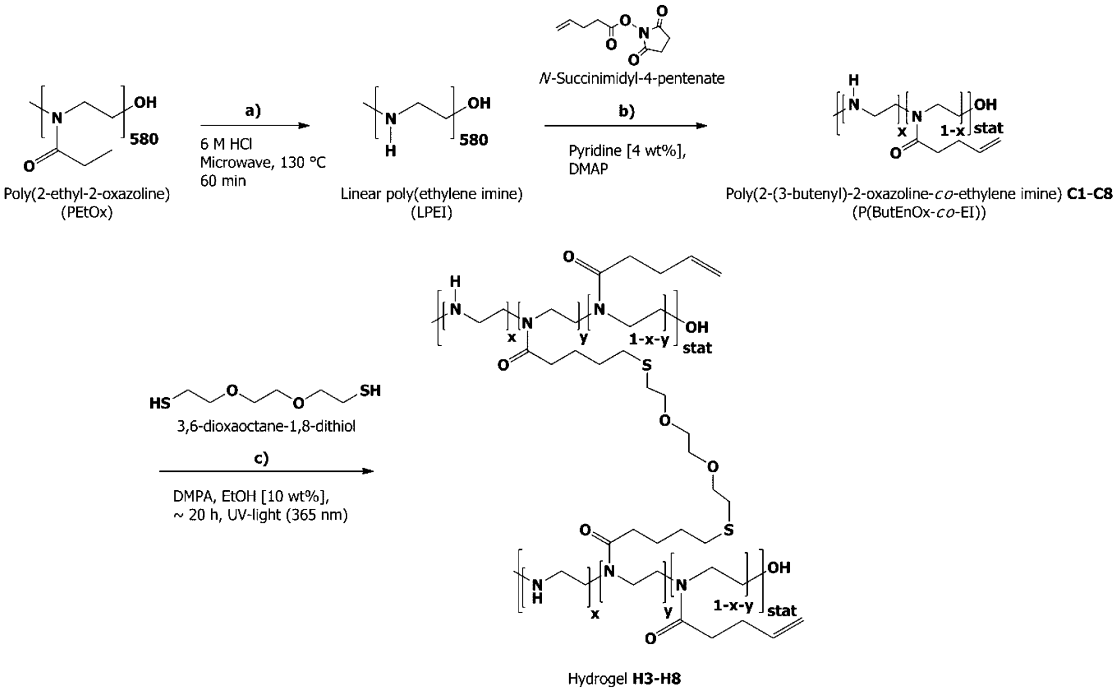
Scheme 1. Schematic Representation of the Synthesis of (a) Linear Poly(ethylene imine) by Acidic Hydrolysis of Poly(2-ethyl-2-oxazoline); (b) Copolymer Poly(2-(3-butenyl)-2-oxazoline-*co*-ethylene imine) Starting from Linear Poly(ethylene imine); and (c) Hydrogel by Cross-Linking the Copolymer via Thiol−Ene Photoaddition



photoinitiator 2,2-dimethoxy-2-phenylacetophenone (0.013 g, 0.05 mmol) and the *bis*-functional thiol, 3,6-dioxaoctane-1,8-dithiol (45 μL, 0.28 mmol), were dissolved in ethanol (0.9:1.0 thiol/double bond). The combined solutions (10 wt %) were degassed with nitrogen for 30 min. Afterward, the clear solution was exposed to UV light (365 nm) for 24 h. The occurring gelation announced the successful synthesis of a three-dimensional network. Subsequently, the obtained gel was washed several times with ethanol (150 mL) and water (150 mL) for 20 min, respectively, and dried by lyophilization.[27,32]

Swelling Value. Q_{eq} 74%. Solid state ^{13}C NMR (100 MHz): δ 173.5, 138.6, 116.4, 71.1, 47.7, 39.4, 32.3 ppm. Solid state ^{1}H NMR (400 MHz, swollen state): δ 5.94 ($HC{=}CH_2$), 5.12 ($HC{=}CH_2$), 4.37−3.25 (N-CH_2, CH_2 EDDET), 3.30−2.07 (CH_2 ButEnOx, CH_2-S-CH_2), 2.02−1.22 (RS-CH_2-CH_2-CH_2) ppm. FT-IR (ATR): 3294 (OH, NH), 2886 (CH asym/sym str), 1636 (C=O), 1421 (C−H def), 1367, 1292, 1234, 1103 (C−N str), 1038 (C−N str) cm^{-1}.

Ethidium Bromide Assay (EBA) of P(ButEnOx-*co*-EI) and Release Studies. The interaction between genomic DNA (gDNA) and cationic copolymers was detected by fluorescence measurements. The EBA was carried out by a procedure adapted from literature.[19] gDNA (7.5 mg mL^{-1}) and EB (0.4 mg mL^{-1}) were dissolved in HBG-buffer (HEPES buffered glucose, pH 7) and incubated for 10 min at room temperature. A total of 100 μL of the gDNA-EB solution were transferred to the wells of a black 96-well plate containing copolymers at defined concentrations (N/P ratios) with different PEI contents. Fluorescence was measured after 15 min of incubation in a repeat determination. A mixture containing only gDNA, EB, and HBG buffer served as calibration standard.

For release studies, 100 μL of the gDNA-EB solution were transferred to a well-plate as described above and the copolymers (3.6 μL of a 1 mg mL^{-1} stock solution, respectively) were added. After incubation, the fluorescence was measured and defined as start value. Subsequently, 10 μL of a heparin stock solution (3 mg mL^{-1}) was added to each of the samples and the fluorescence was measured at defined times.

EBA of P(ButEnOx-*co*-EI)-Based Hydrogels and Release Studies. The hydrogels (1.7 to 2.0 mg) were swollen for 19 h in 250 mL of HBG buffer (pH 7). Subsequently, 1 mL of gDNA-EB solution (containing 7.5 mg gDNA per mL and 0.4 mg EB per mL) was added. The sample aliquot of 50 μL was taken at defined times and returned after fluorescence measurement. Microscopic detection of the resulting fluorescence signal was performed at different time points (0, 1.5, and 18 h).

The release studies were performed by adding 1 mL of a mixture of heparin (6 mg mL^{-1}) and EB (0.4 mg mL^{-1}), dissolved in HBG buffer, to the swollen and loaded hydrogel samples. In addition, the temperature was increased up to 90 °C. Aliquots of 50 μL were taken at defined times and returned after fluorescence measurement.

RESULTS AND DISCUSSION

Synthesis and Characterization of a Copolymer Library of P(ButEnOx-*co*-EI)s. Starting from commercially available poly(2-ethyl-2-oxazoline) (PEtOx, 50,000 g/mol), LPEI was prepared by acidic hydrolysis (degree of hydrolysis > 99%) using a microwave synthesizer (Scheme 1a).[35] Subsequently, LPEI was reacted in an amidation reaction with *N*-succinimidyl-4-pentenate to introduce alkene functionalities into the polymer (Scheme 1b). To this end, LPEI and the catalyst 4-(dimethylamino)-pyridine (DMAP) were dissolved in pyridine at 80 °C and mixed with the activated acid. Due to the negligible effect of the side product *N*-hydroxysuccinimide on the formation of hydrogels, the step of dialysis (performed for 71% PEI containing copolymer) was skipped for further reactions.

Using this method, an eight-membered library of P(ButEnOx-*co*-EI)s (**C1−C8**) with varying amounts of secondary amine groups was synthesized (Table 1).

^{1}H NMR spectroscopy showed the successful conversion of LPEI to the copolymers. The percentage of remaining LPEI units in the copolymer was determined by correlating the integrals of the single proton signal of the double bond (δ = 5.9 ppm, $HC{=}CH_2$, E) to the ones of the LPEI backbone (δ = 3.0 to 2.6 ppm, HN-CH_2-CH_2, B; Figure 1). Moreover, the appearance of the signals of the 4-pentenate protons (δ = 2.6 to

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Table 1. Summary of the Characterization Data for the P(ButEnOx-*co*-EI)s (C1−C8): PEI Content and SEC Data

	C1	C2	C3	C4	C5	C6	C7	C8
PEI [%][a]	85	82	71	62	50	44	36	5
M_n' (NMR) [g·mol^{-1}][b]	32,100	33,500	38,700	43,000	48,700	51,600	55,400	70,100
PDI[b]	-*	-*	1.35	1.37	1.44	1.38	1.64	1.48
M_n (SEC) [g·mol^{-1}][c]	-*	-*	9,400	9,900	10,700	13,700	14,800	14,400

[a]Percentage of remaining LPEI units determined by ^{1}H NMR spectroscopy. [b]Determined by ^{1}H NMR (calculated from LPEI: 25,000 g/mol, PEI content). [c]Determined by SEC (eluent: DMAc + 0.21% LiCl, calibration against polystyrene). *Not soluble in SEC eluent DMAc.

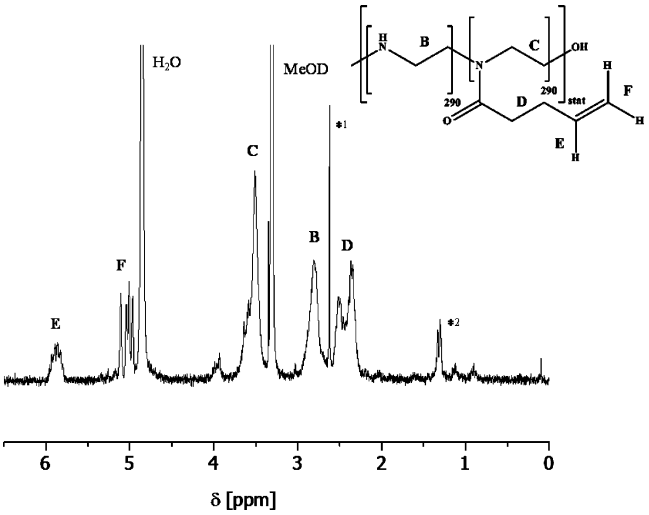


Figure 1. ^{1}H NMR spectrum (MeOD, 250 MHz) of **C5** (50% PEI) produced from linear poly(ethylene imine) (580 units; *1 side-product *N*-hydroxysuccinimide, *2 remaining 2-ethyl-2-oxazoline side chains ~1%).

2.2 ppm, CH_2 ButEnOx, D), the protons of the double bond (δ = 5.0 ppm, HC═CH_2, F) and the oxazoline backbone (δ = 3.8 to 3.4 ppm, RN-CH_2-CH_2, C) prove the proposed structure of the synthesized polymers. The successful conversion was also shown by infrared spectroscopy (IR). After the reaction, a carbonyl vibration of the oxazoline units appears at 1,636 cm^{-1} ($\nu_{C=O}$; Figure 2).

Mass spectrometry (MS) of the copolymers is not possible due to the high molar masses (>32,000 g/mol), which are difficult to transfer to the gas phase. However, using MALDI-TOF-MS, we could obtain a complex isotopic pattern of a low molar mass copolymer (45% PEI content, M_n ~ 1,650 g/mol, ^{1}H NMR: M_n' = 1,800 g/mol). The results are shown in the Supporting Information (Figure S1). Clearly the repeating units of the copolymer can be seen, but due to the complexity of the spectrum, a further assignment is nearly impossible.

Up to a content of 82% PEI, the copolymers are soluble in water. It is known that above a PEI content of 85% the copolymers only dissolve at elevated temperatures.[36] However, all the copolymers showed good solubility in organic solvents such as alcohols (e.g. methanol).

Characterization by size exclusion chromatography (SEC) revealed the formation of polymers with polydispersity indices

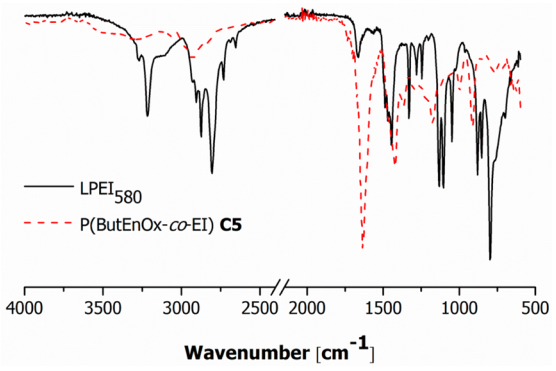
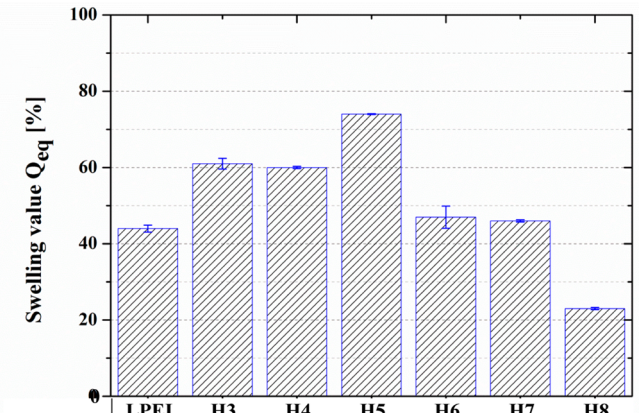


Figure 2. IR spectra of the starting linear poly(ethylene imine) homopolymer and the synthesized copolymer **C5** (50% PEI; determined by ^{1}H NMR spectroscopy of the precursor copolymers).

(PDI values) between 1.35 and 1.64 (Table 1). The molar masses obtained by SEC differ from the molar masses calculated from ^{1}H NMR (M_n'), which are based on the molar mass data of the poly(2-ethyl-2-oxazoline) precursor provided by the supplier. Possible cross-linking or other side reactions are not taken into account. The low molar mass values measured by SEC can be explained considering the different physicochemical properties, that is, the different hydrodynamic volumes of the copolymers and the used PS calibration standard.

Hydrogel Synthesis and Characterization. LPEI-based hydrogels were formed by the reaction of the precursor copolymers P(ButEnOx-*co*-EI)s (**C3−C8**) with 3,6-dioxaoctane-1,8-dithiol (EDDET; Scheme 1c) in a thiol−ene photoaddition reaction. It was performed under UV light using 2,2-dimethoxy-2-phenylacetophenone (DMPA) as initiator. In this way, a library of six hydrogels (**H3−H8**) was synthesized (Figure 3). Due to an insufficient amount of double bonds for cross-linking, copolymers with a PEI content above 82% (**C1**, **C2**) did not form hydrogels at a polymer concentration of 10 wt %, which was adapted from literature.[27] Gelation occurred



	LPEI	H3	H4	H5	H6	H7	H8
PEI [%][a]	100	71	62	50	44	36	5
Q_{eq} [%][b]	*44*	61	60	74	47	46	23

Figure 3. Swelling values Q_{eq} depending on the amount of poly(ethylene imine) units in formed hydrogels **H3−H8** (T = 25.5 °C). Linear poly(ethylene imine) was added as reference (repeated determination).

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after an irradiation time between 150 min for **H3** and 5 min for **H8**. Moreover, an effect of concentration of the prepolymer could be observed. Further information can be found in the Supporting Information (Table S1).

When the reaction was performed under the same conditions but without the dithiol, neither cross-linking nor gelation was observed.

The swelling behavior of the synthesized hydrogels was investigated gravimetrically using centrifuge filter tubes.[19] The filter tubes were saturated with water and the excess water was removed by centrifugation (3,000 rpm, 10 min). The determined mass of the tube was set to m_0. After addition of the hydrogel ($m_{0,gel}$), the sample weight could be determined by eq 1.

$$m_{gel} = m_{0,gel} - m_0 \quad (1)$$

Subsequently, the hydrogel sample was swollen in water for 24 h. The filter tube was centrifuged again (3,000 rpm, 10 min) and weighted (m_{wet}) to determine the mass of the swollen gel (m_{sw}) using eq 2.

$$m_{sw} = m_{wet} - m_0 \quad (2)$$

The swelling value Q_{eq} was calculated according to literature (eq 3).[34]

$$Q_{eq} = \frac{m_{sw} - m_{gel}}{m_{sw}} \times 100\% \quad (3)$$

The formed hydrogels revealed a water uptake up to a multitude of their own mass (Q_{eq} = 23−74%) from the liquid phase. The ability of the formed networks to absorb water is ascribed to the hydrophilic parts of the cross-linker and the oxazoline units. But also the LPEI segments have hygroscopic properties and can exhibit different hydrated states.[37−39] As a reference, pure LPEI (which is insoluble in cold water) was investigated regarding its swelling value (Q_{eq} = 44%).

A maximum of the swelling values of the investigated hydrogels was reached for a PEI content of 50% (**H5**, Figure 3) with 74%. This behavior can be explained with two competing trends. Starting from pure LPEI as reference, a decreasing PEI content, accompanied with an increasing amount of water-soluble gel components, leads to higher swelling values. The decreasing swelling values at LPEI contents lower than 50% can be ascribed to a higher network density, caused by a higher degree of cross-linking for these polymers. The increased linking density and the associated decrease of the degrees of freedom of the polymer chains limit the amount of water, which can be incorporated within the polymer network. This causes the low swelling of the 5% PEI containing gel. Compared to PEtOx-containing hydrogels with similar degrees of cross-linking (Q_{eq} = 97−98%[19]), the swelling values are significantly decreased, which is caused by the lower hydrophilicity of the LPEI systems.

The water uptake behavior from the gas phase was analyzed for **H5** (50% PEI content) at varying humidity levels using a thermogravimetric analysis (TGA) setup, which was already described for the investigation of hydrophilic polymers.[40] The TGA diagram showed the adsorption of water molecules to the hydrogel network as a function of the weight change (%) and the relative humidity (%) at a constant temperature (25 °C). Before starting the measurement, the sample was heated to 60 °C at 0% humidity to completely dry the sample. After the weight of the gel was constant, its mass was set as m_{gel} (compare to eq 1). Subsequently, the relative humidity was gradually increased to 90%, whereupon a weight change of up to 30% (m_{sw}, eq 2) could be observed (equates to Q_{eq} = 23%), with an exponential increase. This value is identical to the swelling behavior of the 5% PEI containing hydrogel determined from the liquid phase. By decreasing the relative humidity in the same way, the desorption curve showed an identical trend, stating a reversibility of the swelling process. In comparison to the swelling studies from the liquid phase (for PEI content >5%), water uptake from the gas phase is much less efficient (about 10×), as depicted in Figure 3.

To confirm the structure of hydrogel **H5** (50% PEI content), solid state NMR measurements were performed. In Figure 4, a

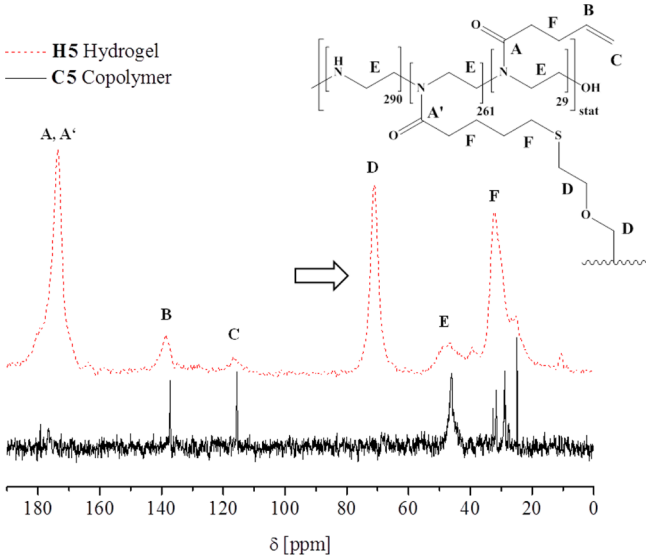

Figure 4. Solid state ^{13}C NMR spectrum of the 50% PEI containing copolymer **C5** (D_2O, 63 MHz) and the resulting hydrogel **H5** (100 MHz).

comparison of the ^{13}C NMR spectrum of the copolymer **C5** and the solid state ^{13}C NMR spectrum of the resulting purified hydrogel **H5** is depicted. An important evidence for a successful synthesis is the appearance of the signal of the cross-linker (D) at 71.0 ppm. In addition, a solid state ^{1}H NMR spectrum was recorded (Figure 5). To improve the resolution, a special method for the sample preparation was used. The gel was swollen for 16 h inside a Kel-F rotor in D_2O to increase the degrees of freedom of the polymer chains, which was placed into the spinning tube. Signals between 2.0 and 1.2 ppm (RS-CH_2-CH_2-CH_2) belonging to the EDDET cross-linker further demonstrated the success of the reaction. Both ^{13}C and ^{1}H NMR spectra revealed an incomplete conversion of the double bonds that, hence, can be used for further functionalization.

DNA Binding Studies. After the successful synthesis of copolymers and hydrogels with well-defined LPEI contents, their ability to bind and release DNA was investigated using an ethidium bromide assay (EBA). Genomic herring DNA was treated with ethidium bromide (EB) resulting in a fluorescent DNA-EB complex, which could be detected using a fluorescence microscope. The assay allowed to monitor the interaction between DNA and polymer/hydrogel by a displacement of EB resulting in a decrease of the fluorescence intensity.[41]

The results of the EBA for the chosen copolymers P(ButEnOx-*co*-EI)s (**C3**−**C5**, **C7**) are depicted in Figure 6a.

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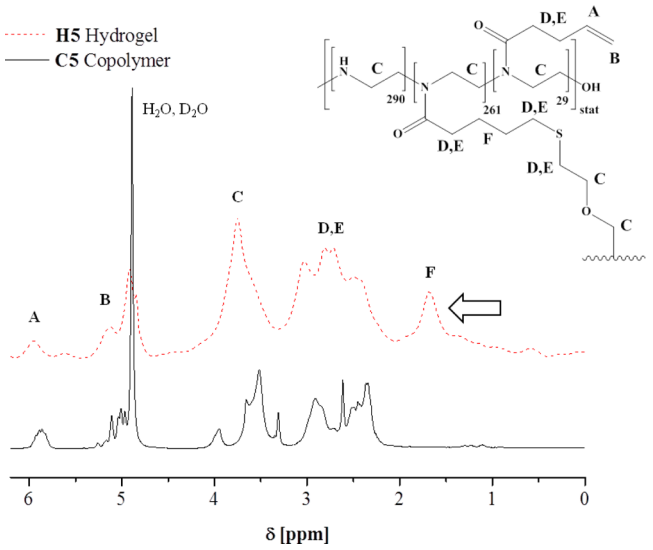

Figure 5. Comparison of the ^{1}H NMR spectrum of the 50% PEI containing copolymer **C5** (MeOD, 250 MHz) and the solid state ^{1}H NMR of the resulting hydrogel **H5** (swollen in D_2O, 400 MHz).

As expected, the system with a high content of LPEI showed an increased binding capacity and, hence, a decreased fluorescence intensity. At a $nitrogen_{polymer}$ to $phosphate_{DNA}$ (N/P) ratio higher than 2, a constant fluorescence level was reached, meaning that further excess of positively charged copolymers was not able to replace more EB. The copolymer with the highest charge density (71% PEI content) showed the highest complexation efficiency of all investigated copolymers, with a decrease in fluorescence intensity of 70%. As reference the LPEI precursor was used, which led to a fluorescence intensity decrease of about 90%.

Three hydrogels with different PEI contents, namely, **H4** (62%), **H5** (50%), and **H7** (36%), were chosen to study their DNA binding and release capability. Due to the high molar masses and the large network structures, low N/P ratios, as used for the copolymers, were not suitable. Hence, the EBA procedure had to be adjusted and N/P ratios between 250 and 500 were investigated (Figure 6b). To obtain comparable results for hydrogels, similar masses were used (1.7−2.0 mg). Prior to the EBA measurements, the hydrogel samples were swollen for 19 h in a 96-well plate in 250 μL of HBG buffer solution, respectively. The large excess of buffer allowed a complete swelling of the hydrogels independent of the PEI content and swelling value. Moreover, the concentration after the addition of 1 mL stock solution to each swollen sample was nearly identical. For each measurement an aliquot of 50 μL of each sample was taken at defined times and returned afterward. A mixture of 250 μL of HBG buffer and 1 mL of stock solution served as reference for each measuring point. To exclude the decrease of fluorescence intensity due to the degradation of the DNA-EB complex, the reference sample was treated and stored exactly as the other samples.

A similar behavior as for the copolymers was observed for the corresponding hydrogels. Besides the decrease in fluorescence intensity over time, a trend dependent on the PEI content was observed. The gel **H4** with the highest amount of PEI units (62%) showed the highest binding capacity with a fluorescence level of 20%, which was significantly lower than the value of the corresponding copolymer. For the 50% PEI containing gel, similar results to the corresponding copolymer were obtained. The hydrogel **H7** with 36% of LPEI showed no DNA binding. A possible explanation is the dense structure of the hydrogel due to the high amount of cross-linking sites hindering the access of DNA to the hydrogel network. This fact also causes the long time required for a complete DNA binding (95 h). Furthermore, the low swelling values and, thus, the inflexibility of the network, led to a time-consuming complexation process. These long adsorption periods might indicate a diffusion controlled process. To sum up, a high PEI content seems to be the predominant factor for high binding capacities of the hydrogels studied as demonstrated by the remarkable value of **H4**. However, the amount of amine groups is limited by the number of functional sides (here alkene groups) necessary for the formation of stable hydrogels. In addition, the DNA binding of the hydrogel sample **H4** was monitored using a fluorescence microscope (Figure 7). The photometric measurements showed the decrease of the fluorescence intensity of the DNA-EB complex over time after the addition of the hydrogel. A DNA-EB solution of the same concentration served as reference and showed no indications of degradation of the dye. In contrast to the DNA investigations of the supernatant of the P(ButEnOx-*co*-EI) copolymers and the corresponding hydrogels, the microscopic study was performed with the complete

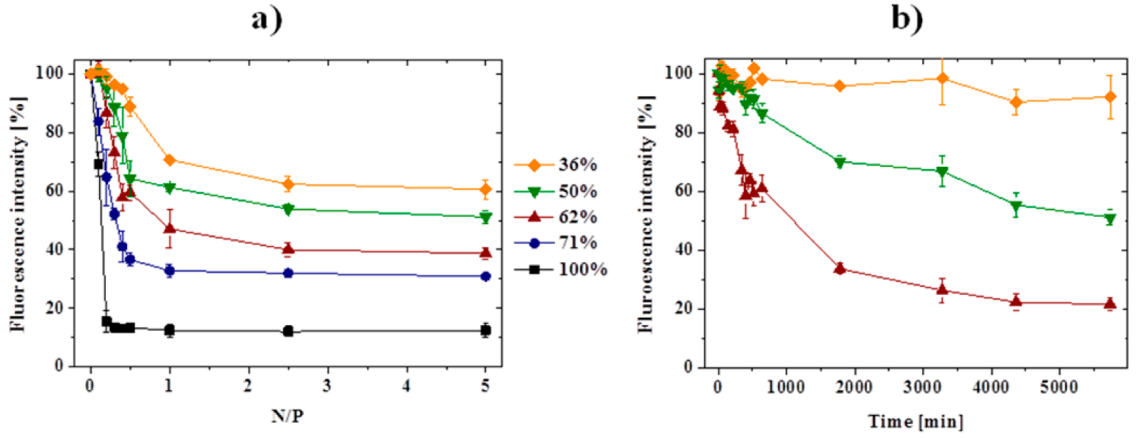

Figure 6. Binding of DNA (ethidium bromide assay)[41] to (a) copolymers **C3**−**C5** and **C7** at increasing nitrogen/phosphate ratios (triple determination) and (b) hydrogel samples **H4**, **H5**, and **H7** at nitrogen/phosphate ratios above 250 (repeated determination) with different PEI content via fluorescence measurements.

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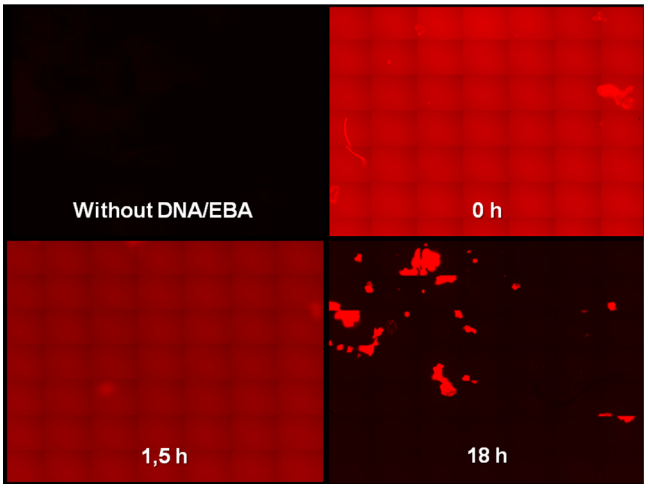

Figure 7. Microscopic pictures of DNA binding to hydrogel **H4** (62% PEI) measured by fluorescence microscopy without DNA/EBA and after addition of DNA/EBA at defined times.

sample including the hydrogel. Since the bound DNA still has free coordination sides for EB, a fluorescence signal is detectable in the area of the hydrogel.

Besides an effective DNA binding, its release from the hydrogel and the copolymer, respectively, represents an essential step for further applications. The DNA release was studied using a heparin assay. Heparin is a polyanion with multiple negative charges per repeating unit, which can effectively bind to the positively charged hydrogel or copolymer, causing the release of the DNA. Thus, the latter can intercalate again with free EB, which was added in the same concentrations as before. The formation of the DNA-EB complex led to an increase of the fluorescence intensity. For the copolymers, the DNA was released very fast. Within 4 min, the fluorescence intensity increased rapidly up to a constant level of nearly 90% (Figure 8a). As reference, a mixture of 250 μL of HBG buffer and 1 mL of DNA-EB stock solution was chosen. It was treated with heparin in the same way as the copolymers, and the resulting fluorescence intensity was set to 100%.

The hydrogels showed a different behavior. Here, neither the addition of heparin (5% release) nor an increase of temperature (up to 90 °C, 10%) resulted in an efficient release. However, a combination of both led to a detachment of nearly 50% of the bound DNA within 80 min (Figure 8b). This behavior could be associated with the PEI segments of the synthesized hydrogels. LPEI is not soluble in cold water and the PEI components of the hydrogels could form clusters within the network that inhibit an efficient release due to sterical reasons. At higher temperatures, the PEI segments melt and a replacement of DNA with heparin becomes possible.

CONCLUSION

In summary, a new and efficient method to obtain hydrogels for the binding and release of DNA with a controlled amount of amine binding sides was developed. To this end, a new class of copolymer containing 2-butenyl-2-oxazoline and ethylene imine units was synthesized by partial functionalization of LPEI with *N*-succinimidyl-4-pentenate. An eight-membered library of P(ButEnOx-*co*-EI)s was prepared, and subsequently, the double bonds were exploited for cross-linking via thiol−ene photoaddition using *bis*-functional 3,6-dioxaoctane-1,8-dithiol. Compared to the formation of PEI hydrogels by cross-linking the free amines, the presented approach allows the exact adjustment of the amount of free amines in the copolymer and, thus, within the hydrogel. Both copolymers and hydrogels were characterized by means of NMR spectroscopy and FT-IR spectroscopy. Furthermore, the swelling and deswelling behavior of the hydrogels from liquid and gas phase was investigated, showing that the water uptake from the liquid phase is 10× more efficient. Swelling values up to 74% were observed.

The DNA studies showed that copolymers and hydrogels are able to bind and release DNA reversibly via the secondary amine groups with binding capacities strongly depending on the PEI content. The copolymers bound up to 70% of the initial DNA. Because of their large three-dimensional networks, the hydrogels exhibited a much higher binding capacity for the appropriate PEI contents. However, due to the low swelling values compared to POx-based hydrogels and, thus, the inflexibility of the network, the DNA uptake was rather slow, probably indicating a diffusion-controlled process.

The DNA release studies were performed using a heparin assay. While the copolymers at room temperature rapidly

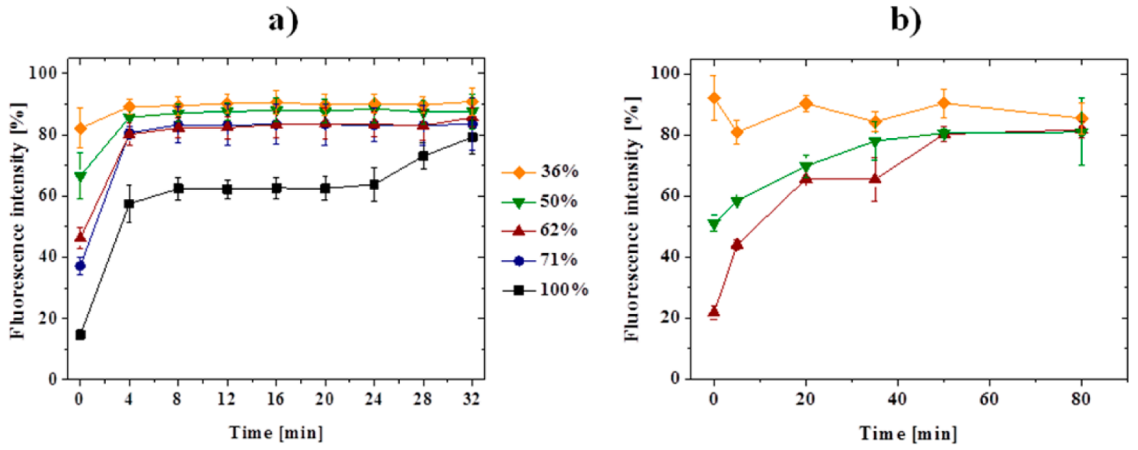

Figure 8. Time-dependent release of DNA from (a) copolymers **C3**−**C5** and **C7** (heparin, triple determination) at a nitrogen/phosphate ratio of 2 and (b) hydrogel samples **H4**, **H5**, and **H7** at nitrogen/phosphate ratios above 250 (heparin, 90 °C, repeat determination) with different PEI content via fluorescence measurements.

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released up to 90% of the initial DNA quantity after heparin addition, the hydrogels released 50% at elevated temperatures.

To accelerate the binding and release of genetic material, further studies, dealing with the improvement of the swelling behavior of the hydrogels, have to be performed. Hence, hydrogels starting from a precursor poly(2-ethyl-2-oxazoline-*co*-ethylene imine) instead of linear poly(ethylene imine) should be investigated. This will be part of a follow-up study.

In addition, solid NMR spectroscopy revealed the presence of unreacted alkene groups, which could be used for further functionalizations like surface attachment. The treated surfaces are available for DNA binding and release studies in terms of chip-based point-of-care diagnostics.

Besides its use in hydrogel synthesis, the copolymer P(ButEnOx-*co*-EI) represents an interesting molecule for other applications, such as gene delivery or gene silencing.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Figure S1 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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