Linear Polyethyleneimine: Optimized Synthesis and Characterization – On the Way to "Pharmagrade" Batches

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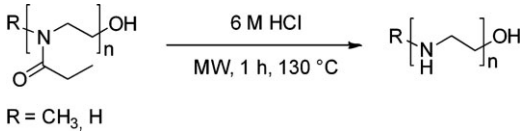
The synthesis of linear polyethyleneimine (LPEI) by acidic hydrolysis of poly(2-ethyl-2-oxazoline) is studied and optimized to reach the highest degree of hydrolysis within the shortest time range using a microwave synthesizer. In addition, the purification procedure is significantly improved; the fast batch processing combined with an excellent control of the actual heating time represents a well-suited alternative to the conventional synthesis on the way to "pharmagrade" PEI. The developed protocol for the preparation of methyl and proton-initiated LPEIs shows a high reproducibility, and the identity and purity of the LPEIs is proven by means of ^{1}H NMR and IR spectroscopy as well as MALDI-TOF- and ESI-Q-TOF-MS.



Introduction

Polyethyleneimines (PEIs) are available in two forms – as branched polyethyleneimine (BPEI) and linear polyethyleneimine (LPEI). Both are of highest interest in pharmaceutical research as polymeric vectors for gene delivery as they can electrostatically interact with negatively charged molecules like DNA and RNA.[1] PEIs with a high molar mass and a high degree of branching demonstrated the formation of small and enzymatically stable polyplexes with nucleic acids with a high cell uptake and therapeutic activity. However, their in vivo and clinical administration were limited due to cytotoxic effects and a low hemocompatibility.[1e] Although LPEIs with comparable molar masses were less effective in condensation of nucleic acids, they were found to be more suitable for in vivo applications due to a reduced toxicity and a higher transfection efficiency compared to the branched form.[2] Some of the commercially available LPEIs (e.g., ExGen500, jetPEI) already reached clinical trials, e.g. for the local treatments of bladder carcinoma and HIV disease.[3]

Beside its important role in gene delivery, PEI has some other applications. Based on its ability to form complexes with anionic species, metal complexes, or metal ions, PEI represents an interesting material for technical applications. On account of its chelating properties, PEI is used in waste water treatment for removing heavy metal ions and in hydrometallurgy for the recovery of noble metals.[4] Another field of application of PEI lays in the paper industry, where it is used for the fixation of pigments, as flocculation aid,[5] and as retention agent to improve the wet strength of paper (Epomin, Polymin).

LPEI can be synthesized by the polymerization of *N*-substituted aziridines and their subsequent hydrolysis.[6] The more common method, though, is to obtain LPEI by the

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acidic[7] or basic[8] hydrolysis of poly(2-oxazoline)s. The living character of the cationic ring-opening polymerization (CROP) of 2-oxazolines allows the preparation of well-defined LPEIs with narrow molar mass distributions. Commercially available LPEIs, e.g. from Polysciences or Polyplus Transfection (jetPEI), are commonly synthesized by acidic hydrolysis of methyl-initiated poly(2-ethyl-2-oxazoline) (PEtOx).[9] However, with reaction times ranging from a few hours up to several days[7c] the acidic hydrolysis of poly(2-oxazoline)s proceeds very slowly. The basic hydrolysis is even slower.[10] More recently, the acceleration of the acidic hydrolysis under microwave irradiation was demonstrated by Schubert et al.[7a] Nevertheless, the reaction conditions were not optimized to reach full hydrolysis to LPEI within the shortest reaction time but rather to prepare partially hydrolyzed poly(2-methyl-2-oxazoline)s and poly(2-ethyl-2-oxazoline)s. Since the degree of hydrolysis significantly influences the transfection efficiency,[1d] a defined and also full degree of hydrolysis is preferred for pharmaceutical and clinical applications. Here, we report the optimization of the hydrolysis conditions of PEtOx using a microwave synthesizer with an autosampler and an improved purification procedure. These techniques were modified on the one hand with regard to reproducibility and controllability. On the other hand, the methods were upscaled to produce PEIs of defined quality in the range of several grams. Moreover, two differently initiated LPEIs, i.e., a methyl- and a proton-initiated, were prepared by acidic hydrolysis of methyl- (**1**) or proton- (**2**) initiated PEtOx. The methyl-initiated LPEI (**3**) contains only secondary amino groups, the proton-initiated LPEI (**4**) exhibits one primary amino group, which allows selective end group functionalization. The differently initiated LPEIs were characterized by proton nuclear magnetic resonance (^{1}H NMR) and infrared (IR) spectroscopy as well as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) time-of-flight (TOF) mass spectrometry (MS). Such an intense structural characterization is essential on the way to "pharmagrade" PEI since up to now only very limited information and protocols, and even less data sheets are available. Consequently, a highly reproducible, time- and yield efficient synthesis protocol allows the preparation of well-defined and in-depth characterized LPEI batches that ensure the absence of impurities and the constancy in structure and, thus, properties.

Experimental Section

Chemicals and Instrumentation

2-Ethyl-2-oxazoline and methyl tosylate were obtained from Acros Organics, distilled to dryness over barium oxide (BaO), and stored under argon. Acetonitrile was purchased from Sigma-Aldrich. An Initiator Sixty single-mode microwave synthesizer from Biotage, equipped with a noninvasive IR sensor (accuracy: ±2%), was used for polymerizations and hydrolyses under microwave irradiation. ^{1}H NMR spectra were recorded on a Bruker AC 300 MHz at 298 K. Chemical shifts are reported in parts per million (ppm, δ scale) relative to the residual signal of the deuterated solvent. ESI-Quadrupole-TOF-MS measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer. The ESI-Q-TOF mass spectrometer was operating at 4.5 kV, at a desolvation temperature of 180 °C, and in the positive ion mode. Methanol or methanol/water mixtures were used as solvents. The ESI-Q-TOF-MS instrument was calibrated in the m/z range 50–3 000 using an internal calibration standard (Tunemix solution), which was supplied from Agilent. The MALDI-TOF-MS spectra were measured on an Ultraflex III TOF/TOF (Bruker Daltonics GmbH, Bremen, Germany). The instrument was equipped with a frequency-tripled Nd:YAG operating at a wavelength of 355 nm. All spectra were measured in the positive reflector mode. For the sample preparation 1 μL of PEI (10 mg · mL^{-1}) and 10 μL of 2,5-dihydroxybenzoic acid (DHB) (10 mg · mL^{-1}), both dissolved in methanol, were mixed. One microliter of this solution was applied to the target using the dried-droplet method. The IR spectra were recorded on a FT-IR spectrometer IRAffinity-1 (Shimadzu). Size-exclusion chromatography (SEC) of the PEtOxs was measured on a Shimadzu system equipped with a SCL-10A system controller, a LC-10AD pump, and a RID-10A refractive index detector using a solvent mixture containing chloroform, triethylamine, and isopropyl alcohol (94:4:2) at a flow rate of 1 mL · min^{-1} on a PSS-SDV-linear M 5 lm column at room temperature. The system was calibrated with polystyrene (370–67 500 g · mol^{-1}) standards.

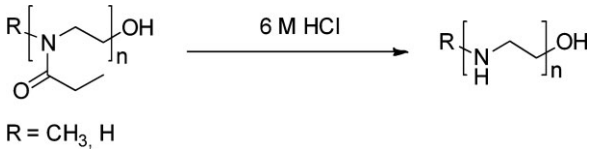
Synthesis of LPEI

The PEtOxs used in this study as starting materials for the preparation of LPEI were synthesized according to literature.[11] The polymerization of 2-ethyl-2-oxazoline was performed at 140 °C in a microwave synthesizer using methyl tosylate or *p*-toluenesulfonic acid as initiator and acetonitrile as solvent. PEtOxs with molar masses between $\overline{M}_n = 1\ 000$ g · mol^{-1} and $\overline{M}_n = 20\ 000$ g · mol^{-1} and low polydispersity index values (PDI ≤ 1.3) were synthesized. As a general method, methyl- (**1**) or proton-initiated (**2**) PEtOxs were treated with an excess of 6 M aqueous HCl for different periods of time at 100 and 130 °C in a flask and a microwave synthesizer, respectively. The acid was removed under reduced pressure, and the residue was dissolved in water followed by the addition of 3 M NaOH until precipitation occurred. The LPEI was filtered off, recrystallized from water, dissolved in methanol or *N,N*-dimethylformamide (DMF), and precipitated into ice-cold diethyl ether. The white precipitate was filtered off and dried in vacuo at 40 °C (methanol) or 60 °C (DMF) for 5 d. The purity and the degree of hydrolysis of the resulting LPEI **3** (methyl-initiated) and **4** (proton-initiated) were determined by ^{1}H NMR spectroscopy.

Optimized Synthesis Protocol

For the production of "pharmagrade" LPEI, the following optimized synthesis protocol was established: The PEtOx **1** or **2** (5 g) was dissolved in 15 mL of 6 M aqueous HCl and heated in a microwave

synthesizer for 1 h. The acid was removed by vacuum distillation (30 mbar) at 130 °C. The residue was dissolved in water (50 mL), and 3 M aqueous NaOH was added until precipitation occurred. The LPEI was filtered off and recrystallized from water (80 mL). After filtration, the LPEI was dissolved in methanol (40 mL) and precipitated into ice-cold diethyl ether (400 mL). The white precipitate was filtered off and dried in vacuo at 40 °C for 5 d. The purity and the degree of hydrolysis of the resulting LPEI **3** or **4** were determined by ^{1}H NMR spectroscopy.



Scheme 1. Schematic representation of the synthesis of the different LPEIs.

Methyl-Initiated LPEI (3)

^{1}H NMR (300 MHz, CD_3OD): $\delta = 3.65$ (t, CH_2-OH), 2.73 (br., $N-CH_2$), 2.39 (s, CH_3-N). IR (FT-IR): $\nu = 3\,217$ (NH), 2 873 (CH_3), 2 804 (CH), 1 446 (CH_2/CH_3), 1 330 (C–N), 1 134 (C–N), 1 103 (C–N) cm^{-1}.

Proton-Initiated LPEI (4)

^{1}H NMR (300 MHz, CD_3OD): $\delta = 3.65$ (t, CH_2-OH), 2.73 (br., $N-CH_2$). IR (FT-IR): $\nu = 3\,271$ (NH_2), 3 217 (NH), 3 116 (NH_2), 2 877 (CH_3), 2 808 (CH), 1 597 (NH), 1 450 (CH_2/CH_3), 1 334 (C–N), 1 103 (C–N) cm^{-1}.

Results and Discussion

Conventional Synthesis of LPEI in a Flask Using Oil-Bath Heating

LPEI can be obtained by acidic[7] or basic[8] hydrolysis of poly(2-oxazoline)s. Since the acidic hydrolysis proceeds much faster than the basic hydrolysis,[10] the acidic treatment was chosen to synthesize LPEI. In a first set of experiments, PEtOx was heated with an excess of 6 M HCl overnight at 100 °C in a flask. LPEIs with two different starting groups, i.e., a methyl group (**3**) and a proton (**4**), and different molar masses were synthesized using this approach (Scheme 1). To the best of our knowledge, it is the first report of a proton-initiated LPEI. The primary amino group renders the possibility for selective end group functionalization. A detailed analysis of the differently initiated LPEIs is feasible by means of ^{1}H NMR spectroscopy (Figure 1). For both types of LPEI, a triplet at $\delta = 1.13$ can be observed. This signal belongs to the methyl group of uncleaved PEtOx side chains. By correlating the integrals of the methyl proton signal of the uncleaved side chain ($\delta = 1.13$) and the protons of the LPEI backbone ($\delta = 2.73$), the degree of hydrolysis can be determined within the limits of the ^{1}H NMR accuracy (98% after 18 h). About 1% of the PEtOx remained uncleaved even by elongation of the reaction time to 26 h. The incomplete hydrolysis is assigned to the precipitation of the LPEI hydrochloride, which is formed during the reaction.

The LPEIs obtained after neutralization with base were soluble in warm water, DMF, and methanol; and insoluble in chloroform, diethyl ether, and dichloromethane. The long reaction times of several hours required an optimization of the reaction conditions in a microwave synthesizer.

Optimized Synthesis of LPEI

The usage of microwave synthesizers is beneficial for the acceleration of the hydrolysis, as already reported.[7a] Based on this previous study, the hydrolysis conditions were further optimized. The usage of a microwave synthesizer enables heating of solvents above their actual boiling points resulting in shorter reaction times. Furthermore, it allows an excellent control of the heating time and rate. Therefore, the synthesis becomes tunable and also highly reproducible, which is important with respect to the pharmaceutical application of LPEI that requires a certified method for the preparation.

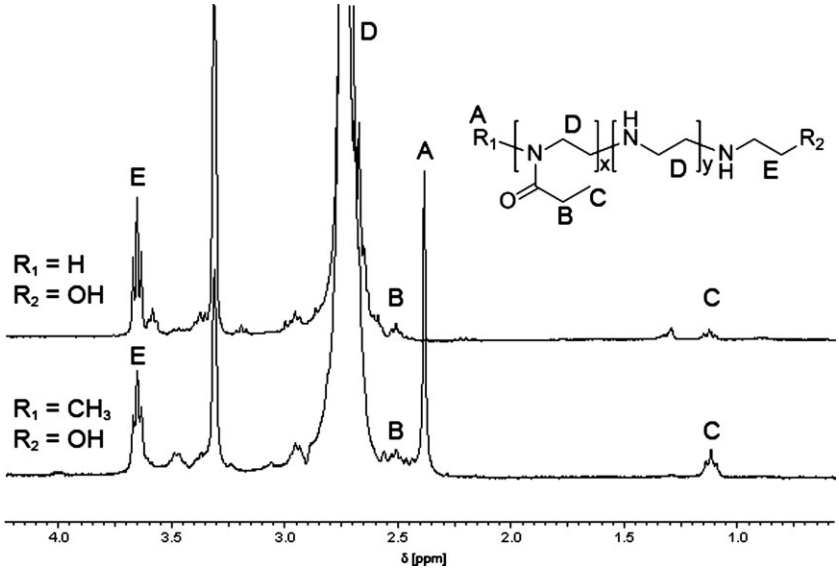


Figure 1. ^{1}H NMR spectra of the different LPEIs (300 MHz, CD_3OD).

Table 1. Degree of hydrolysis of **1** ($n = 20$) under different hydrolysis conditions in a microwave synthesizer.

Entry	Condition [a)]	Time [h]	Temperature [°C]	Degree of hydrolysis[b)] [%]
A	acidic	7	150	99
B	acidic	7	130	99
C	acidic	1	150	99
D	acidic	1	130	99
E	acidic	1/2	130	98
F	basic	1/6	130	12
G	basic	2	130	12

[a)]Acidic: 6 M aqueous HCl, basic: 3 M aqueous NaOH; [b)]Determined by ^{1}H NMR spectroscopy.

For the present study, 100 mg of PEtOx **1** (2 000 g · mol^{-1}, $n = 20$) were dissolved in 4 mL of 6 M HCl and heated in a microwave synthesizer. The degree of hydrolysis of the product was determined by ^{1}H NMR spectroscopy (Table 1). In a first run, compound **1** (2 000 g · mol^{-1}, $n = 20$) was hydrolyzed in a microwave synthesizer for 7 h at 150 and 130 °C (entries A and B), respectively, to yield product **3** (890 g · mol^{-1}, $n = 20$). Both samples showed no difference in their ^{1}H NMR and MALDI-TOF mass spectra, and a degree of hydrolysis above 99% was calculated.

Since the reaction was finished after 7 h, the time dependency of the hydrolysis was investigated in more detail. A test reaction at 150 °C and 1 h reaction time resulted in the same degree of hydrolysis (>99%) as for 7 h (entry C). For the reaction at 130 °C, different hydrolysis times were studied (entries D and E). After 0.5 h 2% of the PEtOx side chains were still uncleaved. Extension to 1 h yielded the maximum degree of hydrolysis (>99%). Longer reaction times did not increase the degree of hydrolysis further. It should be noted that in no event a full PEtOx side chain cleavage could be achieved. Hence, the optimal hydrolysis condition reaching the maximum side chain cleavage within the shortest time is 1 h at 130 °C. Compared to the methods described in literature so far, the reaction time could be significantly reduced.

The optimized microwave-assisted synthesis protocol (130 °C) was used for the synthesis of the LPEIs with molar masses between 430 and 8 600 g · mol^{-1}. The protocol could also be applied for the preparation of proton-initiated LPEI (**4**). In addition, commercially available PEtOx (Aldrich, $\overline{M}_w = 50\,000$ g · mol^{-1}) was hydrolyzed using the microwave-assisted synthesis protocol. 98% of the PEtOx side chains were cleaved within 1 h. Again, prolonged reaction times did not further increase the degree of hydrolysis. However, a subsequent second hydrolysis step increased the degree of hydrolysis slightly from 98 to 99%. Interestingly, commercially available LPEIs exhibit a much lower degree of hydrolysis, e.g. 90% for LPEI 2 500 g · mol^{-1} (Polyscience) and 96% for LPEI 25 000 g · mol^{-1} (Polyscience), according to the ^{1}H NMR measurement protocol described above.

The method established can be applied for a maximum concentration of 0.33 g PEtOx per mL 6 M HCl. At this point, the solubility limit of PEtOx in HCl at room temperature was reached. By using an Initiator Sixty single-mode microwave synthesizer from Biotage, the maximum in reaction volume was limited to 15 mL of stock solution (0.33 g PEtOx per mL 6 M HCl). It is thus possible to hydrolyze 5 g of PEtOx within 1 h, yielding about 2 g of LPEI. The autosampler can handle 24 microwave vials automatically. Due to the limited batch size, the time saving compared to the preparation in the flask is lost in cases of amounts larger than 48 g LPEI to be synthesized. This problem might be overcome by using microwave synthesizers with larger batches or continuous flow microwave reactors. When the concentration was increased to 0.67 g PEtOx per mL of 6 M HCl, the degree of hydrolysis decreased to 87% after 1 h reaction time.

Compared to the fast acidic hydrolysis conditions established above, the basic hydrolysis of PEtOx with 3 M NaOH at 130 °C in a microwave synthesizer proceeded much slower resulting in a degree of cleavage of only 12% after 10 min (entry F). The degree of hydrolysis remained constant even after 2 h reaction time (entry G). This behavior is attributed to the phase separation within the reaction mixture.

Optimized Purification Protocol

For the use of LPEI for polyplex formation in gene delivery, a high purity is essential. In addition, the detailed characterization of the polymer structure, including end group analysis by MALDI-TOF- and ESI-Q-TOF-MS, requires an improved purification procedure. The main impurities are salts. LPEI is known to form complexes with salts (e.g., NaI),[12] which can influence the MS spectra. Thus, the influence of different bases used for the purification of LPEI

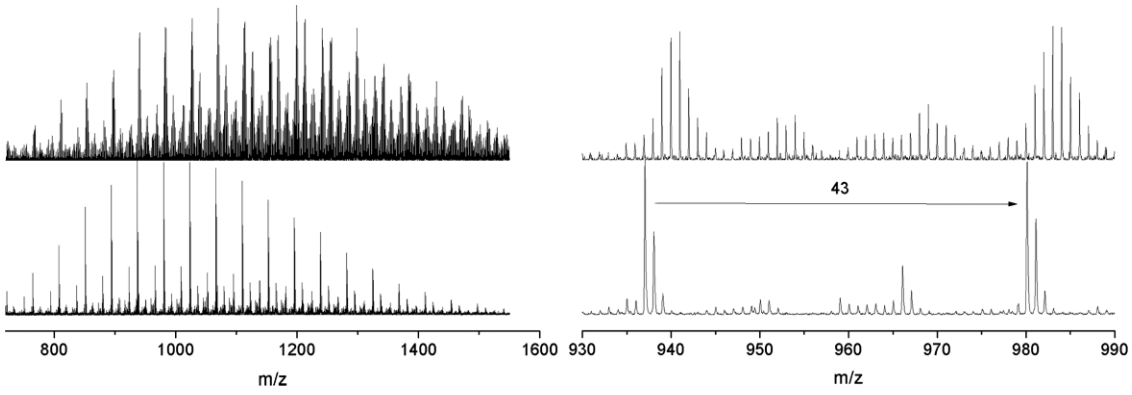


Figure 2. MALDI-TOF-MS spectra of LPEI before (top) and after (bottom) precipitation in diethyl ether (matrix: DHB).

on the resulting MS spectra was investigated, and the purification protocol was optimized.

LPEI was prepared as described above and precipitated using the following bases: NaOH, KOH, LiOH, Cs_2CO_3. Analysis by ^{1}H NMR spectroscopy and MALDI-TOF-MS showed no significant difference between the samples; no influence of the utilized base could be observed. However, the MALDI mass spectra revealed a complex pattern of overlapping peaks preventing a detailed analysis (Figure 2 top). A second recrystallization from water did not improve the quality of the spectra. Nevertheless, for the analysis of differently functionalized LPEIs it is necessary to obtain interpretable MALDI mass spectra for the end group determination. Therefore, the optimization of the purification procedure was investigated.

Since DMF is known to dissolve salts readily, the LPEI was dissolved in DMF and precipitated in a large excess of ice-cold diethyl ether instead of drying directly after the recrystallization from water. The effect of this step on the MALDI-TOF mass spectrum was remarkable (Figure 2 bottom). A disadvantage of this method was the usage of DMF as solvent. High drying temperatures and long drying periods were required to remove DMF completely, even under high vacuum conditions. To overcome this problem, methanol was used to dissolve the LPEI for precipitation in ice-cold diethyl ether. Consequently, the product can be dried in vacuo at 40 °C. ^{1}H NMR spectroscopy as well as ESI-Q-TOF-MS and MALDI-TOF-MS showed no differences to the results of the DMF route. The purity was proven by ^{1}H NMR spectroscopy indicating no solvent signals after drying for 5 d at 40 °C in vacuo.

Characterization of the LPEIs by MALDI-TOF and ESI-Q-TOF-MS

The different LPEIs were characterized by MALDI-TOF as already described for the methyl-initiated LPEI in literature.[7a] In addition, ESI-Q-TOF-MS studies were performed since less fragmentation products, compared to MALDI-TOF-MS measurements, were expected. Caffeic acid (CA) and 2,5-dihydroxybenzoic acid (DHB) without salt addition proved to be the most suitable matrices for LPEIs, as described previously.[13] Although DHB required higher laser intensities, the resolution of the peaks as well as the signal/noise ratio were found to be superior to the results obtained by CA. Hence, all measurements were performed using DHB. For the ESI-Q-TOF-MS measurements, the LPEIs were dissolved in methanol or in a methanol/water mixture.

In both MALDI-TOF and ESI-Q-TOF mass spectra, distributions for the sodium and the proton adduct of LPEI were found. In contrast to the MALDI mass spectra, which showed only singly charged ions, singly (+1) and doubly (+2) charged ionic species appeared in the ESI mass spectra, as depicted in Figure 3. An expanded region of the mass spectra of LPEI **3** ($\overline{M}_n = 860\ g \cdot mol^{-1}$, $n = 20$) is shown in Figure 4. Six of the distributions were assigned to the displayed structures. As expected, proton and sodium adducts of the reaction product could be detected as well as two differently initiated species. The methyl-initiated species derive from the initiation of the polymerization with methyl tosylate. In addition, proton-initiated species, formed by chain transfer reactions occurring during the polymerization of PEtOx, were observed.[14] Moreover, the MALDI-TOF and the ESI-Q-TOF mass spectra showed a species bearing an uncleaved PEtOx side chain. This supports the previous assignment of the triplet at $\delta = 1.13$ in the ^{1}H NMR spectrum. Both MALDI-TOF and ESI-Q-TOF mass spectra showed in principle the same peaks although in the ESI mass spectrum the proton-initiated species in LPEI 3 were hardly visible.

Simple MALDI-TOF and ESI-Q-TOF mass spectra were obtained for the proton-initiated LPEI **4** ($\overline{M}_n = 660\ g \cdot mol^{-1}$, $n = 15$) (Figure 5). Both ESI-Q-TOF and MALDI-TOF mass spectra showed the same peaks. No signals for methyl-initiated LPEI were visible since *p*-toluenesulfonic acid was

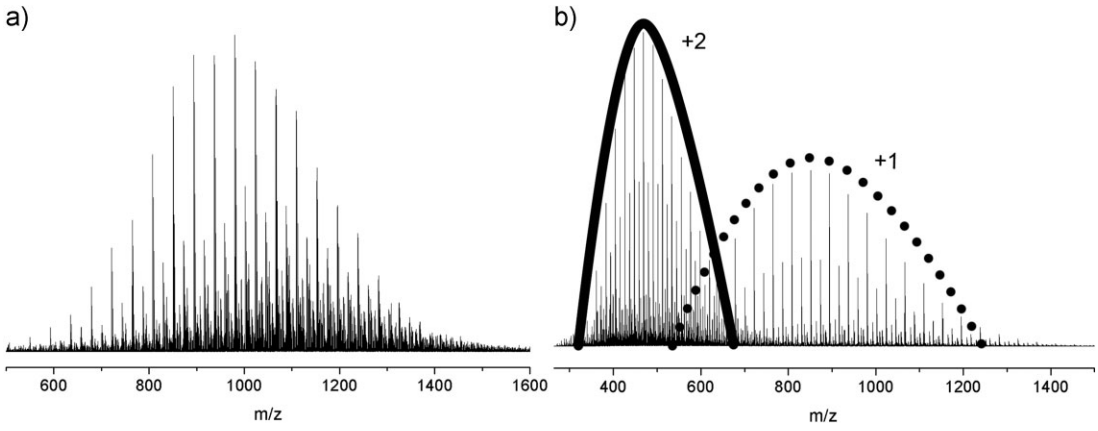


Figure 3. (a) MALDI-TOF-MS and (b) ESI-Q-TOF-MS (straight line: double, dotted line: single charged species) of LPEI **3** (860 g · mol^{-1}, $n = 20$).

used as initiator for the polymerization of the PEtOx. For three of the distributions, corresponding structures could be assigned. In accordance to the spectra of LPEI **3**, a distribution for LPEI with an uncleaved PEtOx side chain was found. The unassigned peaks (mainly in the MALDI-TOF-MS spectrum) belong to fragmentation products. A detailed study on the fragmentation and composition will be published elsewhere.[15] For the hydrolysis performed in both flask and microwave synthesizer, comparable spectra were obtained following the optimized purification protocol.

Since LPEI is a polycation, there is a problem analyzing higher molar masses with ESI-Q-TOF and MALDI-TOF-MS. For high-molar-mass LPEIs, there are numerous multiply charged species, making the ESI-Q-TOF mass spectra very complicated. By means of MALDI-TOF-MS only singly charged species can be detected. Therefore, it is hard to obtain spectra of the multiply charged polycation LPEI, when the degree of polymerization is too large.

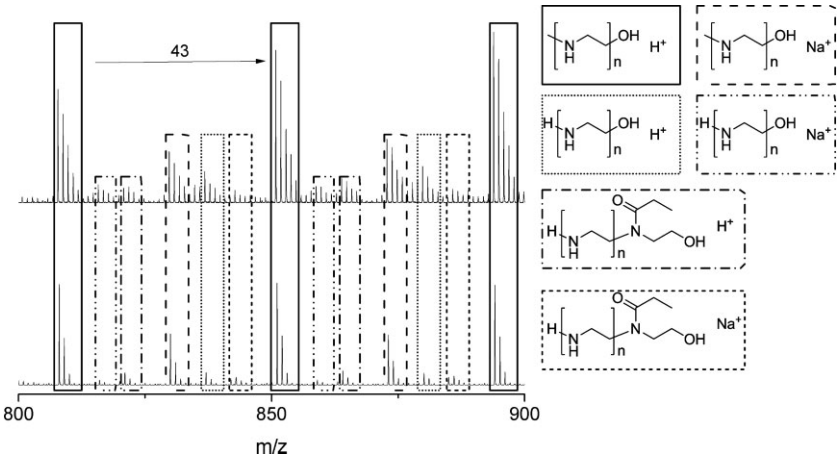


Figure 4. Expanded region of the MALDI- (top) and the ESI-Q-TOF mass spectra (bottom) as well as structural assignments for the different distributions of LPEI **3** (860 g · mol^{-1}, $n = 20$).

Conclusion

In conclusion, both the hydrolysis in a flask and in a microwave synthesizer results in a degree of hydrolysis above 99%. LPEIs with different molar masses (430 g · mol^{-1} to 8 600 g · mol^{-1}) were synthesized using both methods. However, compared to the conventional heating in a flask, the hydrolysis in a microwave synthesizer proceeded much faster. Since the batch processing is automated, it is possible to synthesize larger amounts of LPEI. The fast and efficient hydrolysis and the good heat distribution render the microwave synthesizer to be very energy saving, compared to conventional heating in a flask. Further advantages of a microwave synthesizer are the excellent control of the heating rate and time, making the hydrolysis highly controllable and reproducible. This is important with respect to the development of a certified synthesis method for larger amounts of "pharmagrade" LPEI.

The synthesis conditions were optimized to obtain the maximum of side chain cleavage (99%) within the shortest time range. Although incomplete cleavage (<1%) was obtained even by increasing the reaction time and the temperature, the best and most efficient hydrolysis conditions were found to be 130 °C for 1 h using a microwave synthesizer. The maximum concentration of PEtOx, which can be hydrolyzed, was found to be 0.33 g PEtOx per mL 6 M hydrochloric acid. An increase of the concentration led to a decrease of the degree of hydrolysis.

A methyl and a proton-initiated LPEI were prepared via the improved protocol. Several parameters, which can be used as specification for a "pharmagrade" pro-

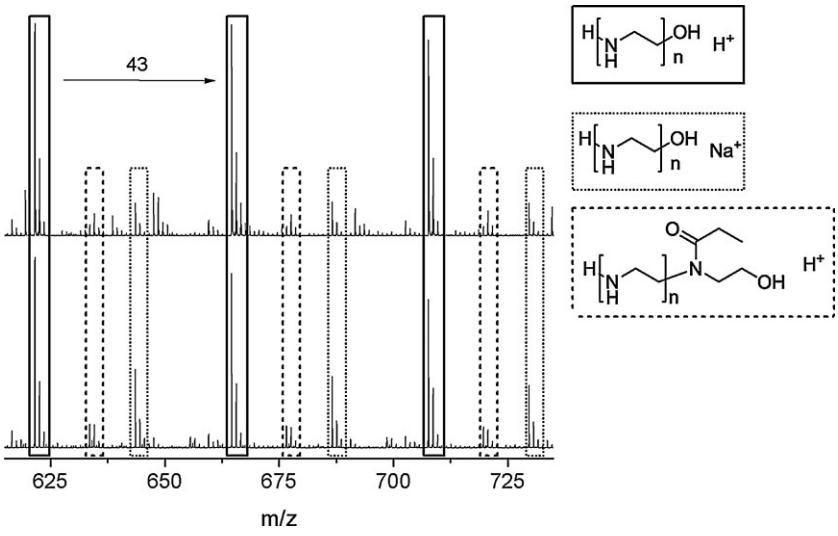


Figure 5. Expanded region of the MALDI- (top) and the ESI-Q-TOF mass spectra (bottom) as well as structural assignments for the different distributions of the proton-initiated LPEI **4** ($\overline{M}_n = 660\ g \cdot mol^{-1}$, $n = 15$).

duction of LPEI, were identified and systematically investigated. The developed protocol showed a high reproducibility, and the identity and purity of the LPEIs was proven by means of ^1H NMR and IR spectroscopy as well as MALDI-TOF- and ESI-Q-TOF-MS. To the best of our knowledge, this contribution contains the first report of a proton-initiated LPEI. The primary amino group of **4** enables the possibility of a selective end group functionalization of the LPEI.

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