Synthesis, Characterization, and Gene Transfer Application of Poly(ethylene glycol-*b*-ethylenimine) with High Molar Mass Polyamine Block

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The study of ethyloxazoline/methyloxazoline (EtOXZ/MeOXZ) copolymerization, initiated by methyl tosylate (MeOTs), showed that (i) incorporation of MeOXZ units into random copolymer becomes effective over DP = 100 and (ii) propagation process proceeds with negligible transfer to monomer up to a DP of 400 despite the presence of MeOXZ in the polymerization medium. These results produced random poly(EtOXZ-*co*-MeOXZ) copolymers with various molar composition ratios in alkyloxazoline units. The close values found for the comonomer reactivity ratios in acetonitrile ($r_{1MeOXZ} = 1.18$; $r_{2EtOXZ} = 0.34$) implied a random chain organization in short sequences of each repeating unit, which was an important parameter in view of the optimization of their subsequent modification: the alkaline hydrolysis was successfully achieved when the MeOXZ unit content of the polyoxazoline chains reached 75%. Using these results, the diblock copolymer poly(ethylene glycol-*b*-(ethyloxazoline-*co*-methyloxazoline)) (poly(EG-*b*-(EtOXZ-*co*-MeOXZ))) with high DP was synthesized by cationic copolymerization of EtOXZ/MeOXZ comonomers using $CH_3-PEG_{2kDa}-Ts$ as macroinitiator. The comonomer composition of this new compound was adjusted in order to optimize the hydrolysis step and obtain finally the diblock copolymer poly(ethylene glycol-*b*-ethylenimine) (poly(EG-*b*-EI)). The high molar mass of this copolymer was confirmed both by ^1H NMR and SANS measurements. Gene delivery experiments showed that the copolymer has significant DNA transfection capacities.

Introduction

Cationic polymers such as polyethylenimines (PEIs) and particularly the 22 kDa linear polyethylenimine ($LPEI_{22kDa}$), which contains around 500 monomer units, belong to the most effective synthetic vectors for DNA transfection of eukaryotic cells.[1] However, the capacity of these compounds to deliver genes into the nucleus of primary cells or to transfect in vivo tissues other than lung after systemic administration is limited.[1,2] In addition, the solubility, biodegradability and chemical homogeneity of these polymers are not satisfactory. Therefore, design and synthesis of new vectors remain an important approach. One way to increase the in vivo half-life of transfection particles and to improve the solubility of DNA complexes consists of conjugating poly(ethylene glycol) (PEG) residues to the cationic polymer.[3,4] Such conjugates are mostly obtained by grafting PEG residues to the PEI. Interestingly, DNA condensation with these PEGylated PEIs was noted to be less efficient than that with nonmodified PEIs.[4,5] This is likely to be due to the hydrophilic PEG arms which hinder efficient binding of the cationic polymer to DNA. Furthermore, in vitro cell binding and uptake of PEG-PEI/DNA versus PEI/DNA complexes is also decreased, indicating that PEGylation reduces the interactions of the polyplexes with the target cells.[4] It is therefore not surprising that the in vitro transfection efficiency of such PEG-PEI conjugates is significantly lower than that of the corresponding nonmodified polymer.[4] To circumvent these problems, we decided to synthesize a diblock poly(ethylene glycol-*b*-ethylenimine) (poly(EG-*b*-EI)) copolymer of high molar mass polyamine block.

The synthesis of a such compound with DP 80 for the LPEI block was previously reported by Akiyama et al.[6] It was carried out in two steps: synthesis of a poly(2-alkyloxazoline) (PROXZ) precursor block by cationic polymerization of 2-methyloxazoline monomer (2-MeOXZ) initiated by PEG macroinitiator, followed by alkaline hydrolysis of the PMeOXZ segment. However, in these conditions, the DP of the polyamine block is severely limited due to chain transfer occurring during polymerization of the MeOXZ monomer.[7] Therefore, this synthetic route cannot be applied to access to copolymers with high molar mass LPEI block.

The new strategy developed in the present work consisted in the use of the more polymerizable ethyloxazoline (EtOXZ) monomer in order to obtain a DP higher than 100 for the polyalkyloxazoline (PROXZ) block. Therefore, the cationic copolymerization of either EtOXZ monomer alone or a mixture of EtOXZ/MeOXZ comonomers was successively investigated in high DP conditions, using respectively the α-methoxy *ω*-*p*-toluenesulfonyl poly(ethylene glycol) ($CH_3-PEG_{2kDa}-Ts$) and methyl tosylate (MeOTs) electrophilic initiators. The paper describes also how to obtain complete hydrolysis of the PROXZ sequences of these copolymers by circumventing the problems linked both to PEG block sensitivity in acidic medium[8] and the low solubility of the poly(EtOXZ) chains in basic media.[9] Finally, we evaluated the transfection activity of the synthetic DNA carrier poly(EG-*b*-EI) of high DP.

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Experimental Section

Materials. Anhydrous ethanol (SDS), NaOH pellets (Aldrich), acetone (SDS), methyl tosylate (Aldrich), and diethyl ether were used as received. MeOXZ (Aldrich), EtOXZ (Aldrich), and acetonitrile (SDS) were distilled over calcium hydride. The linear PEI 22kDa and α-methoxy-ω-4-toluenesulfonyl poly(ethylene glycol) ($CH_3-PEG_{2kDa}-Ts$) were synthesized as previously described.[10,11]

SMD2-LucΔ*I*TR (7.6 kbp) is an expression plasmid encoding the firefly *luciferase* gene under the control of the human cytomegalovirus (CMV) immediate-early promoter.

Polymer Characterization. Polymer analysis was performed by NMR spectroscopy (Brucker 300 MHz, solvents: $CDCl_3$, CD_2Cl_2); SEC (Waters System, columns: set of PL aquagel OH30 and OH40, eluent: water + $LiNO_3$ 0.1M + NaN_3, detector MiniDawn LS (Wyatt Technology), laser $\lambda = 690$ nm, scattering angles 45°, 90°, and 135°, calibration of columns poly(ethylene oxide) standards with a range of M_W from 200 to 64 5000); small-angle neutron scattering (SANS) measurements were performed in the Orphee reactor in Saclay on a PACE spectrometer. To cover a wide *q*-range, two configurations (distance from sample to multidetector/wavelength) were used: 4.7 m/13 Å (low-*q* configuration) and 2.5 m/6 Å (large-*q* configuration). The experimental *q* range was $3 \times 10^{-3} < q$ (Å^{-1}) $< 1.4 \times 10^{-1}$ ($q = (4\pi/\lambda \cdot \sin\theta/2)$. All the samples were prepared in D_2O/DCl (0.5 M), NaCl (1 M). The samples were loaded into Hellma quartz neutron cells with a 2 mm optical path length. The scattering for each sample was measured for about 3 h at 20°C. The scattering length densities of solvent and poly(EG-*b*-EI) (**9**) have been calculated assuming density equal to 1.1 for **9** ($n_s = +6.4 \times 10^{10}$ cm^{-2} and $n_{\mathbf{9}} = +0.9225 \times 10^{10}$ cm^{-2}).

Synthesis of Poly(EtOXZ-*co*-MeOXZ) Random Copolymers. As an example, polymer **3** was prepared using the following procedure: methyl tosylate (106 mg, 0.57 mmol) was dissolved in 73 mL of dried acetonitrile. MeOXZ (9.1 mL, 107 mmol) and EtOXZ (10.7 mL, 108 mmol) were then added, and the solution was stirred at 82 °C. A small portion of the reaction mixture was sampled out at different times and purified by precipitation in diethyl ether. For each sample, the copolymer yield was determined and used to calculate the monomer conversion versus time which then allowed us to draw the Ln M_0/M_t = f(t) plot. ^{1}H NMR (CD_2Cl_2): δ (ppm) = 7.6 and 7.1 (d, TsO^-); 3.4 (m, CH_2-CH_2-N-CO); 2.3 (m, $N-CO-CH_2-CH_3$); 2.0 (t, $N-CO-CH_3$); 1.0 (b s, $N-CO-CH_2-CH_3$). ^{13}C NMR (CD_2Cl_2): δ (ppm) = 174.5 ($N-CO-CH_2-CH_3$); 171.3 ($N-CO-CH_3$); 46.5 (CH_2-CH_2-N-CO); 26.5 ($N-CO-CH_2-CH_3$); 21.7 ($N-CO-CH_3$); 10.0 ($N-CO-CH_2-CH_3$).

To determine the reactivity ratios, five experiments with different molar feed ratios of comonomers were carried out and all stopped at low yield. For example, to a solution of methyl tosylate (102 mg, 0.55 mmol) in acetonitrile (14 mL), MeOXZ (6.8 mL, 79.9 mmol) and EtOXZ (2.7 mL, 27.3 mmol) were added, and the reaction mixture was stirred at 82 °C. After 15 min, 10 mL of solution were sampled and evaporated under reduced pressure to remove the excess of monomers, and the comonomer composition of the collected polymer was determined by ^{1}H and ^{13}C NMR.

Synthesis of Poly(EG-*b*-EtOXZ) and Poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) Diblock Copolymers. As a representative example, the synthesis of polymer **5** was prepared as follows: 710 mg of $PEG_{2kDa}-Ts$ (Mn = 2100 g/mol, 0.3 mmol) were dissolved in dried acetonitrile (46.5 mL) before addition of MeOXZ (33.5 mL, 0.39 mol) and EtOXZ (20 mL, 0.19 mol). The solution was stirred for 96 h at 82 °C, then concentrated under reduced pressure, and precipitated by dropwise addition of diethyl ether. After filtration, the product was dried in vacuo (18% yield). ^{1}H NMR (CD_2Cl_2): δ (ppm) = 7.6 and 7.1 (d, TsO^-); 3.6 (s, CH_2-CH_2-O); 3.4 (m, CH_2-CH_2-N-CO); 2.3 (m, $N-CO-CH_2-CH_3$); 2.0 (t, $N-CO-CH_3$); 1.0 (b s, $N-CO-CH_2-CH_3$). ^{13}C NMR (CD_2Cl_2): δ (ppm) = 174.8 ($N-CO-CH_2-CH_3$); 171.6 ($N-CO-CH_3$); 71.2 (CH_2-CH_2-O); 46.6 (CH_2-CH_2-N-CO); 26.6 ($N-CO-CH_2-CH_3$); 21.7 ($N-CO-CH_3$); 10.0 ($N-CO-CH_2-CH_3$).

Synthesis of Poly(EG-*b*-EI) Diblock Copolymer 9. To a solution of poly(ethylene glycol-*b*-(ethyloxazoline-*co*-methyloxazoline)) **5** (1.5 g, 0.04 mmol) in water (30 mL) were added NaOH pellets (2.6 g, 65 mmol). The reaction mixture was heated under reflux for 53 h. After cooling, the copolymer precipitated spontaneously. The product was then filtered and washed with water. Yield was 717 mg (93%). ^{1}H NMR (CD_2Cl_2): δ (ppm) = 3.6 (s, CH_2-CH_2-O); 2.7 (bs, CH_2-CH_2-NH); 1.9 (bs, N*H*).

Cell Culture. Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) was supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% of foetal calf serum (FCS). Human hepatocarcinoma cells (HepG2 cells; ATCC) and transformed human embryonic kidney cells (HEK293; ATCC) were used for the transfection experiments.

DNA Retardation Assay. DNA binding was studied by means of an agarose gel retardation assay. Plasmid DNA (1 μg) and increasing amounts of polymer **9** were each diluted in 25 μL of 150 mM NaCl and mixed. After 15 min of incubation, samples (20 μL) were electrophoresed through a 1% agarose gel using Tris-borate-EDTA buffer. The DNA was visualized after ethidium bromide staining.

Transfection Experiments. Cells plated in 24-well plates were transfected once confluency reached 50−80%. DNA complexes were generated as follows: 4 μg of plasmid DNA and the desired amount of polymer **9** (0.5 mg/mL solution) were each diluted in 100 μL of 150 mM NaCl and gently mixed. After 15 min of incubation the mixture was diluted with serum-free medium to a final volume of 1 mL, and then 0.5 mL was transferred into each well of the duplicate. After incubation for 3 h at 37 °C, the medium was removed and replaced with fresh one containing 10% FCS. Luciferase activity was measured 30 h after transfection.

For luciferase activity, cells were harvested in 250 μL of lysis buffer (8 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1% Triton X-100, 15% glycerol, and 25 mM Tris-phosphate buffer pH 7.8). The cell lysate was then transferred into Eppendorf tubes and centrifuged for 5 min at 10000 g to pellet debris. Luciferase activity was measured in a 96-well plate format with a luminometer (Perkin-Elmer) from an aliquot of the supernatant (50 μL). The measurement was done over 10 s after automatic injection of 100 μL assay buffer (lysis buffer without Triton X-100 supplemented with 2 mM ATP) and 100 μL of luciferin solution (167 μM in water; Molecular Probes). Luciferase background was subtracted from each value and the transfection efficiency was expressed as total light units/10 s/well and are the means of duplicates. The protein content of the transfected cells was measured by Bradford dye-binding using the BioRad protein assay.

Cell Viability Assay. Cytotoxicity was evaluated by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay. In short, 1 day after transfection, the cell culture medium was removed and replaced by serum-free DMEM containing 0.5 mg/mL MTT. After incubation at 37 °C for 4 h, the medium was removed, and 200 μL of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable cells. Absorbance was then measured at 570 nm. Untreated cells were used as control.

Size and ζ-Potential. DNA (25 μg) and 62.5 μg of poly(EG-*b*-EI) **9** were each diluted in 625 μL of either glucose (5%) or NaCl (150 mM) solution. After the solutions were mixed and left for 15 min at room temperature, particle size and ζ-potential were measured using a Malvern Zetasizer nano ZS.

Solubility of Poly(EG-*b*-EI)/DNA Complexes Versus LPEI/DNA. Complexes were prepared in 5% glucose with increasing amounts of DNA, but by keeping the w/w ratio constant (1/1 and 3/1 ratio for LPEI and **9**, respectively). The solubility of the polyplexes was determined by measuring the turbidity of the solution at 602 nm. The absorption of LPEI and poly(EG-*b*-EI) in the absence of DNA was subtracted from the OD values.

Table 1. Polymerization of 2-EtOXZ (M) Initiated by $CH_3-PEG_{2KDa}-Ts$ (I) in Acetonitrile to 82 °C

run	$[I]_0$ ($\times 10^2$ mol/L)	$[M]_0$[a] (mol/L)	time (h)	yield (%)	DPn_{theo}[b]	DPn_{EtOXZ}[c]	Mn_{NMR}[d]	Mn_{SEC}[e] (*I*p)
1a	1.22	1.50	143	100	120	130	15000	14500 (1.2)
1b	1.04	1.50	311	100	270	320	34000	24900 (1.2)
1c	0.89	1.50	488	98	440	460	48000	24000 (1.2)
1d	0.75	1.50	695	78	590	680	70000	29000 (1.2)

[a] Experiment using "IMA" technique with addition of the same volume of monomer than the sample out from the preceding experiment. [b] Total DP of the polyoxazoline chain determined from the yield after polymerization of each increment added following the relation $\sum([M]_0/[I]_0 \times \text{yield})$. [c] Determined by ^1H NMR assuming one PEG block per chain. [d] Calculated from DPn_{EtOXZ} using the molar masses of PEG and EtOXZ units. [e] SEC in H_2O, light scattering detection.

Results and Discussion

Synthesis of Poly(EG-*b*-ROXZ) Copolymer with High Molar Mass PROXZ Block by Polymerization of EtOXZ. The chemical procedure considered for the synthesis of the poly(EG-*b*-ROXZ) copolymer must provide both a polyoxazoline (PROXZ) block of high DP and its subsequent hydrolysis in alkaline conditions avoiding PEG degradation.[8] Consequently, the use of 2-phenyloxazoline (2-ϕOXZ) and 2-methyloxazoline (2-MeOXZ) monomers was not suitable because the segment PϕOX is not soluble in basic aqueous medium, and polymerization of 2-methyloxazoline cannot give DPs exceeding 100 because of chain transfer.[7] Therefore, we attempted to synthesize long blocks of polyalkyloxazoline by polymerization of 2-ethyloxazoline (EtOXZ) with $CH_3-PEG_{2kDa}-Ts$ macroinitiator (previously synthesized and described in reference[11]), using incremental monomer addition technique (IMA) and sampling out from time to time in order to follow the reaction course. After the workup procedure, the total theoretical polymerization degrees (DPn_{theo}) of the PROXZ part of the copolymers poly(EG-*b*-EtOXZ) collected at different reaction times were estimated from their yield (Table 1).

On the basis of the ratio *R* of peak areas corresponding respectively to methylene protons of PEtOXZ block ($-CH_2CH_2N$-(COEt)$-$) at 3.4 ppm and of PEG ($-CH_2CH_2O-$) at 3.6 ppm, the ^1H NMR analysis in $CDCl_3$ of the four copolymers **1a**−**1d** allowed to determine the DP of their poly(EtOXZ) block ($DPn_{EtOXZ} = R \times 45$ (DPn of PEG_{2kDa})). The rather good agreement obtained between DPn_{theo} and DPn_{EtOXZ} in this experiment (Table 1) shows an efficient incorporation of monomer with time after each increment added which implies that the oxazolinium centers remain active during time.

The average molecular weight of the compounds were also determined by SEC analysis using light scattering detection in order to circumvent some unspecific interaction of the polar polymer chains, such as polyalkyloxazolines or PEG, with the column material.[12] Despite this, the Mn values of all copolymers were found lower than those expected from ^1H NMR (Table 1). This discrepancy could be attributed to some adsorption or the existence of transfer reaction during polymerization process. In the first case, the ratio Mn_{NMR}/Mn_{SEC} has to be constant with monomer conversion, while it must increase if transfer occurred. Now, we found a 2-fold increase of the ratio Mn_{NMR}/Mn_{SEC} from the compound **1b** to **1d**. This result suggests that some transfer to monomer began when DP is over 300 for the PEtOXZ chains, during the polymerization of EtOXZ.

The copolymer **1c** of rather high molar mass and DPn_{EtOXZ} was then tested to react in aqueous alkaline conditions. Unfortunately, the hydrolysis gave poor results due to medium heterogeneity following coalescence (see Table 3).[9] To avoid this difficulty, it was decided to introduce more easily hydrolyzable MeOXZ monomer units into the polyoxazoline block of the copolymer.

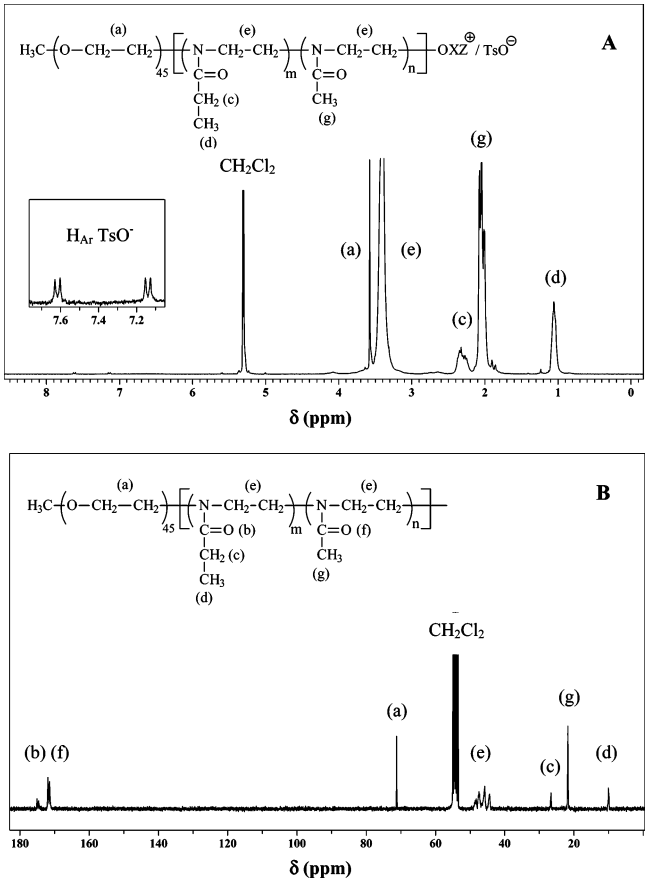


Figure 1. 300 MHz ^1H NMR (A) and 75 MHz ^{13}C NMR (B) spectra of poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) **5** in CD_2Cl_2.

Synthesis of Poly(EtOXZ-*co*-MeOXZ) Based Copolymers. We investigated the synthesis and characterization of random poly(EtOXZ-*co*-MeOXZ) and block poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) copolymers with a DP around 500 for the PROXZ block. To this end, the copolymerization study of EtOXZ/MeOXZ comonomers using respectively MeOTs and $CH_3-PEG_{2kDa}-Ts$ as initiators was carried out. After polymerization, the crude copolymers were precipitated in diethyl oxide, and the NMR analysis of ether layers revealed that they did not contain any trace of oligomers, initiator or macroinitiator.

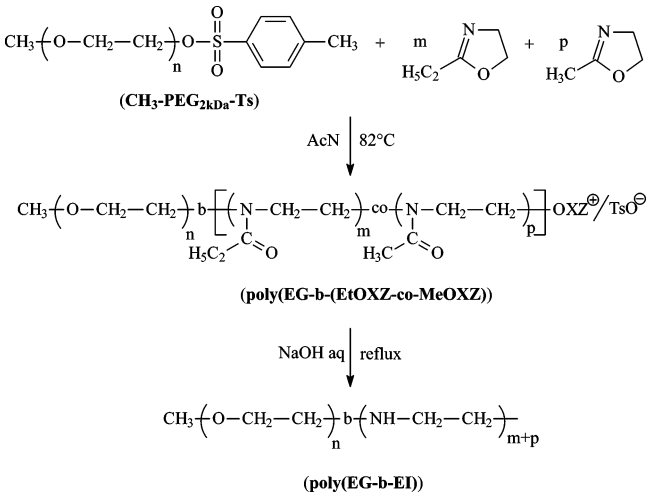
The ^1H NMR spectra in CD_2Cl_2 of all copolymers, analyzed in the aromatic protons part, confirmed the lack of unreacted initiator (no signal of tosyl ester group at 7.3 and 7.8 ppm) and the presence of characteristic tosylate group peaks corresponding to counterion of oxazolinium chain ends at $\delta = 7.1$ and 7.6 ppm (Figure 1A). This result implies a successful initiation even with $CH_3-PEG_{2kDa}-Ts$. These signal integrations, with those corresponding to respective acyl side groups of MeOXZ and EtOXZ repeating units at 2.0 and 1.0 ppm, were used to estimate the polymerization degrees of comonomers (DPn_{MeOXZ} and

Table 2. Copolymerization of 2-EtOXZ/2-MeOXZ Initiated by MeOTs or $CH_3-PEG_{2KDa}-Ts$ in Acetonitrile to 82 °C

run	$[ROXZ]_0$[a]/$[I]_0$	$[MeOXZ]_0/[EtOXZ]_0$	time (h)	yield (%)	DPn_{theo}[b]	DPn_{MeOXZ}[c]	DPn_{EtOXZ}[c]	Mn_{NMR}[d]	Mn_{SEC}[e] (Ip)
2[f]	630	0.6	55	73	460	200	250	42000	19400 (1.5)
3[f]	380	1	73	95	360	200	160	33000	19800 (1.3)
4[f]	750	2	94	72	540	430	140	51000	23400 (1.4)
5[g]	1980	2	96	18	360	310	90	37000	13000 (1.3)

[a] Total initial concentration of comonomers ($[MeOXZ]_0 + [EtOXZ]_0$). [b] DP of the polyoxazoline chain determined from the yield following the relation $[ROXZ]_0/[I]_0 \times$ yield. [c] Determined by ^1H NMR spectroscopy assuming one tosylate group per chain. [d] Calculated from DPn_{EtOXZ} and DPn_{MeOXZ} using the molar masses of MeOXZ and EtOXZ units. [e] SEC in H_2O, light scattering detection. [f] MeOTs initiator used. [g] $CH_3-PEG_{2KDa}-Ts$ initiator used.

Scheme 1. Synthetic Route of Poly(EG-*b*-EI) Using $CH_3-PEG_{2KDa}-Ts$ as a Macroinitiator



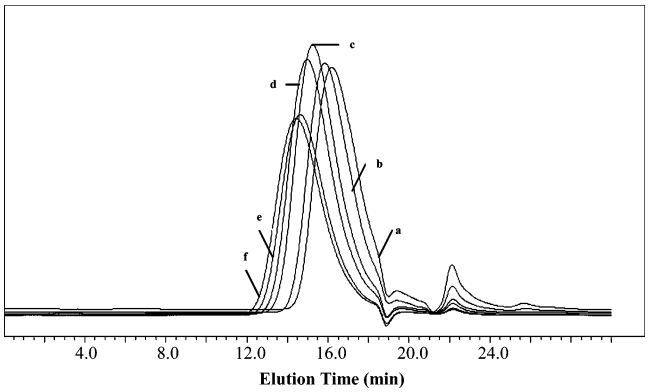


Figure 2. Molecular weight distribution vs time in copolymerization of 2-EtOXZ/2-MeOXZ initiated by MeOTs in acetonitrile to 82 °C (cf. run **3** Table 2); (time (h), polymer yield (%), Mn_{NMR} (g/mol), Mn_{SEC} (g/mol), and (Ip) for samples: (a) 8, 11, 4900, 5600 (1.1); (b) 12, 18, 6800, 4700 (1.2); (c) 24, 38, 13600, 7800 (1.2); (d) 32, 51, 18500, 11000 (1.2); (e) 51, 88, 32000, 18300 (1.2); (f) 73, 95, 33000, 19800 (1.3)).

DPn_{EtOXZ}) and consequently the composition of the random polyoxazoline sequences of polymers (Table 2). The results showed a fair agreement between DPn_{theo} and the total NMR DP of the respective monomer units, which seems to indicate that the random copolymerization process can produce polyoxazoline (PROXZ) chains of total DP around 500 and containing more than 100 MeOXZ units. Of note, since MeOXZ monomer unit content rises with increasing $[MeOXZ]_0/[EtOXZ]_0$ ratio, the synthesis of random PROXZ chains with varying composition in the range $0.8 < DPn_{MeOXZ}/DPn_{EtOXZ} < 3.4$ was available by this way.

According to these results, the diblock poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) copolymer **5** was synthesized with an adjusted comonomer composition allowing optimization of its subsequent basic hydrolysis to obtain the desired poly(EG-*b*-EI) vector (Scheme 1). Of note, a 3-fold higher feed ratio $[M]_0$total/$[I]_0$ was introduced in this experiment to compensate for the decrease of polymerization rate induced by the use of PEG macroinitiator compared to MeOTs.[11] The peak area of PEG block at 3.6 ppm allowed to confirm the total DP of 400 found for the random polyoxazoline sequence of the polymer **5** by using the aromatic protons part (Table 2; Figure 1A). Further, the determination by ^{13}C NMR of the peak intensities ratio of either respective carbonyl groups of MeOXZ at 171.5 ppm (f) to those of EtOXZ at 174.8 ppm (b), or of the carbons at the α position of carbonyl group in amide units respectively at 21.7 (g) and 26.6 ppm (c), confirmed the ratio DPn_{MeOXZ}/DPn_{EtOXZ} found to be 3 by ^1H NMR (Table 2; Figure 1B).

Despite the use of light scattering detection, the SEC analyses of the copolymers containing both EtOXZ and MeOXZ amide units gave again lower molecular weights than those expected from NMR (Table 2). Thus, to acertain the existence of transfer in random copolymerization due to the use of large amounts of MeOXZ monomer, the analysis of molar mass distribution over formation of copolymer **3** was done by sampling out. The SEC profiles showed that its molecular weight increased regularly with monomer conversion (i.e., with time) until the end of polymerization (Figure 2). Moreover, since the value of the ratio Mn_{NMR}/Mn_{SEC} was constant (1.7) for the high molar mass samples of the series (**3**c−**3**f), this indicates that no transfer reaction to monomer occurred up to a DP_{theo} about 400 during copolymerization.

The above conclusion involved that the poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) **5** contained only a few random polyoxazoline chains. Moreover, due to the lower efficacy of PEG macroinitiator compared to MeOTs in initiating the process,[11] this copolymer contained both short and long chains. Despite a little low Mn_{SEC} value, the high molar mass of this compound was, however, evidenced after hydrolysis of the product, by recovering in very quantitative yield the diblock poly(EG-*b*-EI) with high DP, as shown farther by neutron scattering (SANS).

Determination of Kinetic Parameters in the Copolymerization of 2-EtOXZ/2-MeOXZ Comonomers Initiated by MeOTs. The kinetic study of 2-EtOXZ/2-MeOXZ copolymerization initiated by MeOTs was performed on run **2** (Table 2) in order to determine the propagation rate constant of the system. Small portions of polymer solution were sampled out from time to time, the solvent and unreacted comonomers eliminated in vacuo and the resulting polymers precipitated in diethyl oxide before ^1H NMR and SEC analyses. As shown in Figure 3, the linear plot obtained for $Ln([M]_0/[M]_t = f(t)$ indicates that the propagating process is of first order with respect to monomer. Consequently, since the SEC analysis showed an increase of molar mass with time during random copolymerization (Figure 2), this implies that growth takes place on all chains until the end of reaction.

The absolute propagation rate constant value determined from the slope, $kp = 2.2\ 10^{-3}\ mol^{-1}\cdot L\cdot s^{-1}$, was close to that earlier reported for the MeOTs/MeOXZ polymerization system (kp =

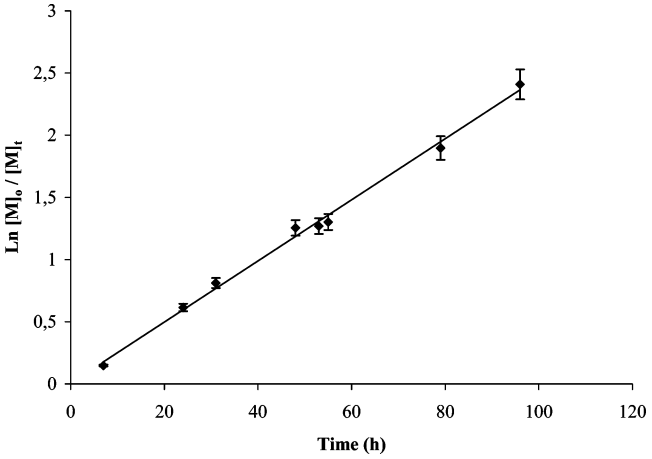


Figure 3. Plot of the kinetic equation Ln ($[M]_0/[M]_t$) vs time in the copolymerization of 2-EtOXZ/2-MeOXZ initiated by MeOTs in acetonitrile to 82 °C (run **2** Table 2).

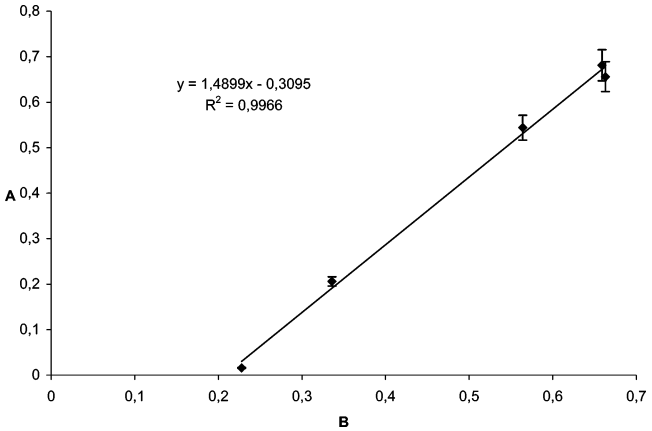


Figure 4. Determination of MeOXZ and EtOXZ monomers reactivity ratios by Kelen−Tüdos method: $A = G/(\alpha + F)$; $B = F/(\alpha + F)$; $\alpha = 1.09$. r_{1MeOXZ} was determined from the A value for $B = 1$ and r_{2EtOXZ} from the $B = 0$ intercept corresponding to $-r_2/\alpha$.

$2.9\ 10^{-3}$ $mol^{-1}\cdot L\cdot s^{-1}$) and indicated a slightly smaller reactivity for EtOXZ monomer.[13]

The determination of EtOXZ and MeOXZ monomer reactivity ratios was then achieved. To this end, five experiments with different feed ratios $[MeOXZ]_0/[EtOXZ]_0$ of comonomers were carried out and stopped for a yield below 10% in order to assimilate the concentration of unreacted comonomers to their initial concentration. After the workup procedure (cf. exp part), the yield and DPn_{MeOXZ}/DPn_{EtOXZ} composition of the polymers recovered were determined as described above, allowing to estimate the ratio d[MeOXZ]/d[EtOXZ] of comonomers consumed in each run. From the well-known equations of the Kelen-Tüdos method (Supporting Information S1), the linear plot $A = f(B)$ obtained with the five experiments gave access to the reactivity ratio of each monomer (Figure 4). The experimental values found in acetonitrile for r_{1MeOXZ} (1.18) and r_{2EtOXZ} (0.34) and the product $r_1 \times r_2$ lower than 1 at the same time showed (i) a slightly higher reactivity for MeOXZ than EtOXZ toward both oxazolinium active species and consequently (ii) the presence of short sequences of each monomer unit in the poly(EtOXZ-*co*-MeOXZ) chains.

Synthesis of Poly(EG-*b*-EI) Diblock Copolymer with a High Molar Mass LPEI Block. The hydrolysis of alcanoyl groups (ethanoyl and propanoyl) of repeating amide units in the poly(EtOXZ-*co*-MeOXZ)-based copolymers containing various MeOXZ unit contents was achieved in concentrate alkaline aqueous medium to reflux (Table 3). At the end of reaction, the polymers **6** and **7** (coming from compounds **1c** and **2**), which contained a large amount of amide units because of low hydrolysis contents, were soluble in the medium at room temperature and therefore isolated, after elimination of water, by dissolution in dichloromethane in order to eliminate excess of NaOH and sodium alcanoate (ethanoate and propanoate) insoluble in organic solvent. On the opposite, the polymers LPEI **8** and poly(EG-*b*-EI) **9** fully hydrolyzed precipitated spontaneously in the medium and were recovered by filtration. This workup procedure eliminates free PEG when present as mentioned in the literature.[6,14] On the ^1H NMR spectra in CD_2Cl_2 of all polymers collected, we observed at 2.7 ppm the characteristic peak of methylene units ($-CH_2-CH_2-NH-$) formed during hydrolysis of amide moieties. The hydrolysis content was evaluated by the integrations ratio of this peak to those corresponding of all $-CH_2-CH_2-N-$ (amine and amide units) present at 2.7 and 3.4 ppm. The results showed that the extent of hydrolysis increases with the MeOXZ units content and became optimal when this value reached 75% in the polyoxazoline block (Table 3).

Concerning the precipitated block copolymer poly(EG-*b*-EI) **9** coming from hydrolysis of poly(EG-*b*-(EtOXZ-*co*-MeOXZ)) **5**, the NMR spectrum revealed a complete hydrolysis by the disappearance of alcanoyl (ethyl and methyl side groups) proton peaks at 1.1, 2.0, and 2.4 ppm, simultaneously with a shift of methylene protons signal of backbone polymer from 3.4 to 2.7 ppm. This new peak observed, together with that of PEG present at 3.6 ppm, confirmed the obtention of the diblock polymer as previously reported.[6,14] The DP of the LPEI sequence of compound **9**, determined by NMR from the molar composition ratio of repeating units in PEG (DP = 45) and polyamine blocks, was found about 700, i.e., higher than that of the PROXZ block of the starting polymer **5**. This result can be explained by the loss of 7% (w/w) of poly(EG-*b*-EI) chains with LPEI sequence of low DP (determined to be lower than 30 by NMR) in aqueous layer, when recovering the compound. Further, since SEC analysis was not possible with this compound because of adsorption, an evaluation of the average molecular weight of the diblock copolymer was done by neutron scattering experiments.

Small-Angle Neutron Scattering Analysis (SANS) of Poly(EG-*b*-EI) Copolymer. The analysis of poly(EG-*b*-EI) by SANS technique allowed to determine its weight-average molar mass.[15] Figure 5A shows the SANS scattering intensity $I(q)$ as a function of q obtained from copolymer **9** in D_2O/DCl (0.5 M), NaCl (1 M). The scattering intensity obtained at large q values was related to the conformation of the chain. Thus, the asymptotic behavior at this q range gave us a power law, $I \sim q^{-0.8}$, which is in tune with a rigid rod like structure for the polymer due to the repulsion effect of neighboring positive charges. For the determination of the size of the molecules, the scattering intensity can be approximated by Zimm eq 1 at small q values:

$$\frac{1}{I} = \frac{1}{I_0}\left(1 + \frac{R_g^{\,2}}{3}q^2\right) \qquad (1)$$

as shown in Figure 5B, where the linear part was used to extrapolate the scattering intensity I_0 at q close to zero. The molecular weight of copolymer **9** was calculated according to eq 2

$$M_w = \frac{I_0 \times d^2 \times N_A}{(n_{\mathbf{9}} - n_s)^2 \times C} \qquad (2)$$

Table 3. Hydrolyzis of Poly(EtOXZ-*co*-MeOXZ) Based Copolymers in Alkaline Medium to Reflux

	starting copolymer		reaction conditions		resulting copolymer	
run	*N*°	MeOXZ content (%)	[NaOH]/[N-COR][a]	time (h)	extraction content (%)	hydrolyzis content (%)
6	**1c**[b]	0	2.3	113	91	14
7	**2**[c]	44	2.6	44	88	29
8	**4**[c]	75	4	90	97	99
9	**5**[d]	77	4	53	93	97

[a] Total N−COMe and N−COEt amide units concentration. [b] Poly(EG-*b*-EtOXZ). [c] poly(EtOXZ-*co*-MeOXZ). [d] Poly(EG-b(EtOXZ-*co*-MeOXZ)).

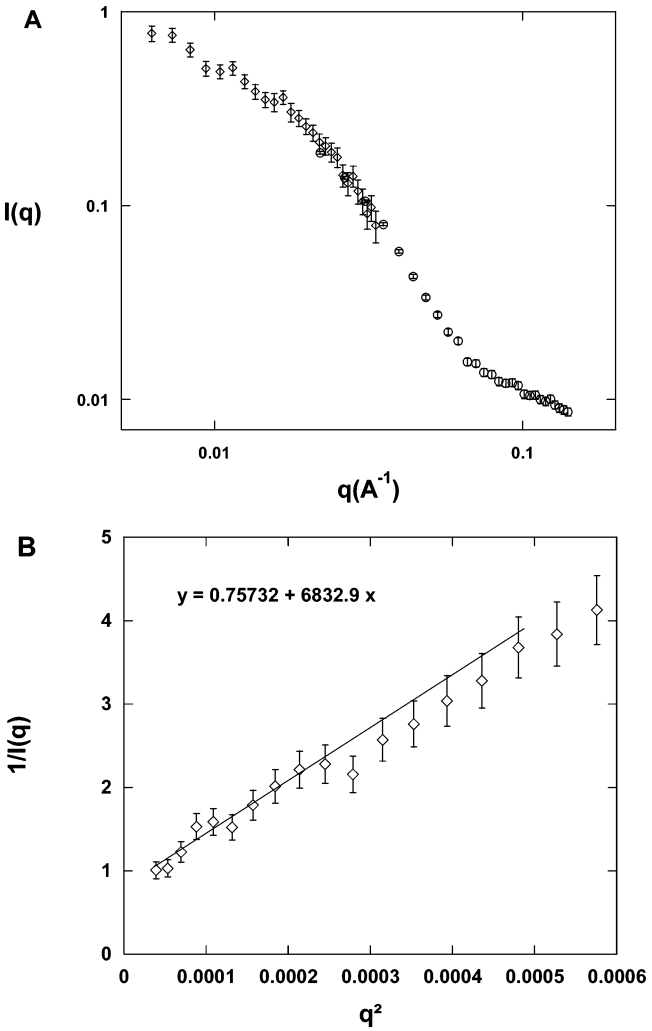


Figure 5. (A) SANS intensities of the copolymer poly(EG-*b*-EI) **9** at concentration 6.32 mg/mL in D_2O/DCl (0.5M)/NaCl (1M). Diamonds correspond to low-*q* configuration and circles to large-*q* configuration. (B) Zimm plot of polymer **9** in D_2O/DCl (0.5 M)/NaCl (1 M).

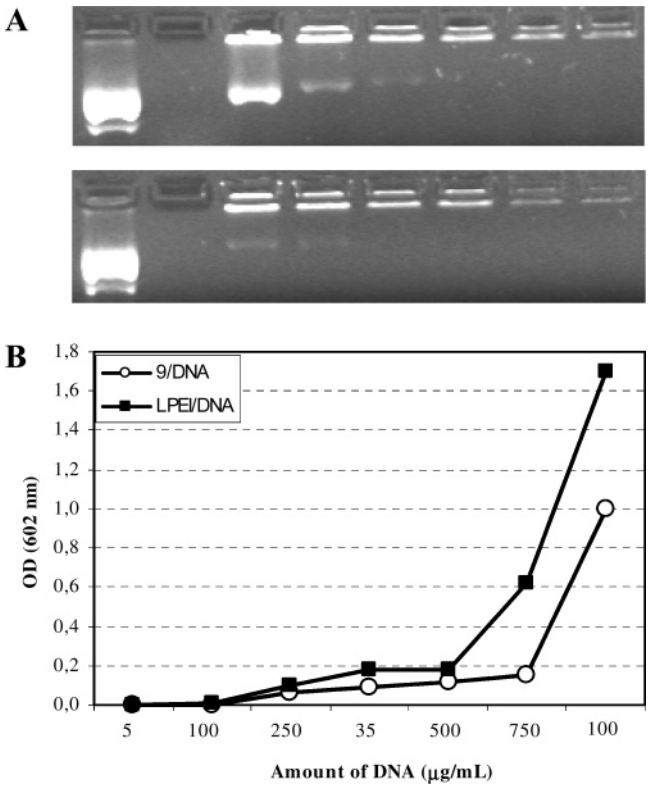


Figure 6. (A) Evaluation of the capacity of the copolymer poly(EG-*b*-EI) **9** to complex plasmid DNA. DNA binding was studied by means of an agarose gel retardation assay. Plasmid DNA (1 μg) and increasing amounts of poly(EG-*b*-EI) (upper gel) or LPEI (lower gel) were each diluted in 25 μL of 150 mM NaCl and mixed. After an incubation of 20 min, samples (20 μL) were electrophoresed through a 1% agarose gel. Lanes 1−8: (1) naked DNA, (3−8) complexes prepared with 0.25, 0.5, 0.75, 1, 2, 3.5 μg of polymer, respectively. (B) Solubility of LPEI/DNA and **9**/DNA complexes was determined in a salt-free solution (5% glucose; see Experimental Section for details).

where *d* and *C* are, respectively, the density and the concentration of **9**, N_A the Avogadro number, n_9 and n_S the scattering length densities of **9** and the solvent, respectively.

The apparent molecular mass was found to be $M_w = 51000 \pm 5000$ g/mol in D_2O/NaCl/DCl for the copolymer with all amino groups protonated, which corresponds to a DP of about 600 for the LPEI block. Although this result is only indicative, it is nevertheless rather in good agreement with that found from NMR.

Gene Transfer Study. Among the different copolymers that were synthesized, we were particularly interested in compound poly(EG-*b*-EI) **9** which possesses at the same time a LPEI and a PEG block.

We first assayed the ability of copolymer **9** to interact with plasmid DNA under physiological ionic strengths (150 mM NaCl). The magnitude of the carrier/DNA interactions was evaluated by the amount of copolymer required to retard the migration of plasmid DNA toward the cathode during agarose gel electrophoresis. As shown in Figure 6A, at a w/w ratio of 1, the copolymer completely inhibited DNA migration (upper gel). At higher ratios, DNA was hardly visible, indicating that DNA is sufficiently well compacted to limit its access to ethidium bromide. For comparison, the LPEI 22 kDa completely retarded DNA migration at a polymer to DNA ratio of 0.75/1 (w/w) (Figure 6A lower gel).

The transfection efficiency of copolymer **9** was tested on human hepatocarcinoma cells (HepG2) and transformed human embryonic kidney cells (HEK293). Therefore, increasing amounts of polymer were added to 4 μg of a plasmid encoding a *luciferase* gene and applied to the cells. After 30 h, the levels of luciferase were measured. As control, we used the LPEI of 22 kDa, which was previously shown to be highly efficient in vitro.[10] The results shown in Figure 7A,C indicate that the

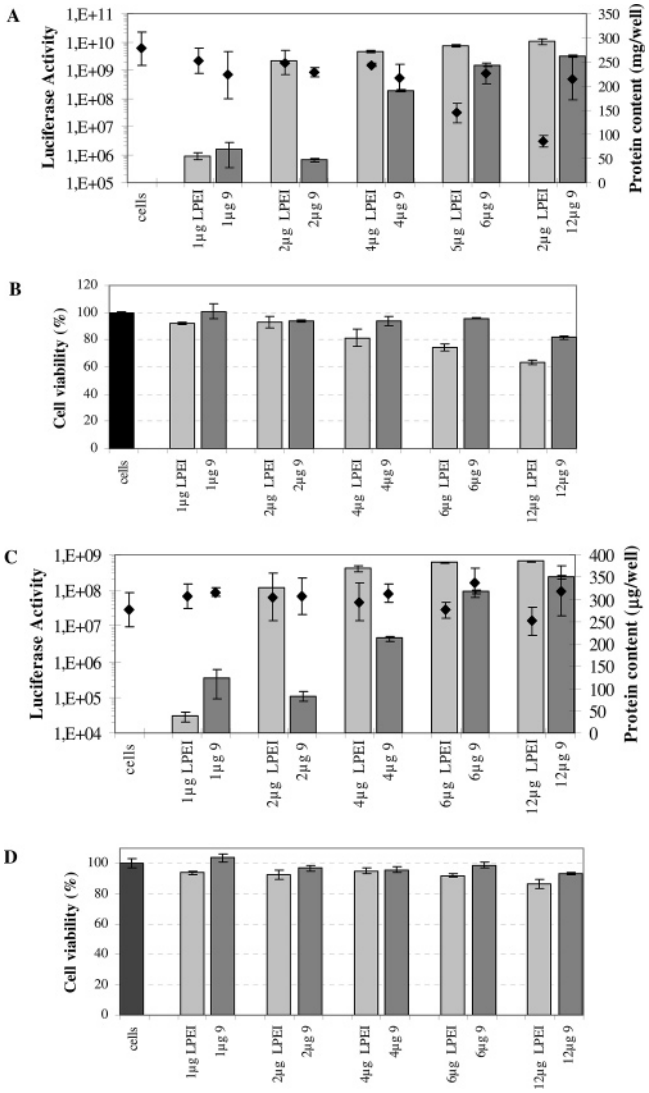


Figure 7. Evaluation of the transfection efficiency of poly(EG-*b*-EI) **9**. Increasing amounts of poly(EG-*b*-EI) and LPEI were mixed with a constant amount of reporter gene (4 μg per duplicate). The complexes were incubated in a serum-free culture medium for 3 h with HEK293 (A, B) or HepG2 (C, D) cells. The luciferase activity was measured 30 h post-transfection. The transfection efficiency was expressed as total light units/10 s/mg protein and is the mean of the duplicates (A, C). The protein content (black squares) was determined by using the BioRad protein assay. At the same time, the MTT cell viability assay was performed on both cell lines as described in the methods section (B and D).

copolymer allows for efficient gene transfer in both cell lines. In fact, the levels of reporter gene expression obtained with the diblock polymer are comparable to those obtained with the nonmodified PEI although higher w/w ratios were required with the former compound. The toxicity of both types of formulations was evaluated by measuring the protein content as well as by performing a MTT cell viability assay. The results obtained by both methods indicate that at the concentration giving the highest transfection, the two polymers have comparable cytotoxicities (Figure 7). Of note however, when using higher concentrations, in particular on HEK293 cells, the diblock copolymer is less toxic than its nonmodified counterpart.

The size and the surface charge (ζ-potential) of DNA complexes are parameters that play an important role in the cell uptake efficiency. Therefore, we measured these two characteristics for copolymer **9**/DNA complexes. Of note, it was previously shown that the size of transfecting particles changes

Table 4. Size and ζ-Potential of Copolymer Poly(EG-*b*-EI)/DNA Complexes[a]

	size (nm)	
time (min)	5% glucose	150 mM NaCl
15	70	1065
60	70	1752
120	69	3701
	ζ-Potential (mV)	
15	+18	+6

[a] 62.5 μg copolymer/25 μg DNA/1.25 mL.

depending on the buffer used to generate the DNA complexes. For example, the linear PEI of 22 kDa generates small DNA particles (diameter < 100 nm) when the complexes are prepared in 5% glucose. In contrast, in the presence of 150 mM of sodium chloride, the size of the particles is micrometric.[16] Therefore, the size measurements of our complexes were performed by using either a salt-free (5% glucose) or a salt-rich (150 mM NaCl) solution. Our results show that, as with the LPEI 22 kDa, the size of copolymer/DNA complexes dramatically increased in the presence of salts (Table 4). Of note, the size of the complexes generated in glucose did not change with time. As expected, the surface charge of the complexes prepared at a ratio effective for in vitro transfection was found to be positive, independent of whether they were generated in salt-free or salt-rich conditions.

To determine whether the coupling of a hydrophilic PEG chain results in an increased solubility, we prepared polyplexes with LPEI and **9** by keeping the N/P ratio constant but by increasing the amount of DNA from 50 to 1000 μg/mL. The turbidity of the solution was followed by measuring the OD. The results show that polyplexes containing the copolymer are more soluble than LPEI/DNA complexes (Figure 6B). This is the case although **9** was used at a 3/1 w/w ratio and LPEI at a 1/1 ratio.

Conclusion

It has been demonstrated that the incorporation of methyloxazoline (MeOXZ) monomer units with polymerization degrees higher than 100 into poly(alkyloxazoline)s chains could be obtained by copolymerization with ethyloxazoline (EtOXZ). Moreover, the determination of comonomer reactivity ratios and propagation rate constant for the copolymerization initiated by methyl tosylate (MeOTs) showed that this system has a kinetic behavior close to that of MeOTs/MeOXZ, but proceeds without transfer up to a DP of 400. These results were used for the synthesis of a diblock copolymer poly(EG-*b*-EI) with high molar mass LPEI block. The new copolymer was successfully synthesized by cationic polymerization of 2-EtOXZ/2-MeOXZ comonomers initiated by α-methoxy-ω-4-toluenesulfonyl poly(ethylene glycol) macroinitiator, followed by the basic hydrolysis of random poly(ethyloxazoline-*co*-methyloxazoline) (poly(EtOXZ-*co*-MeOXZ)) segment. The high molar mass of the diblock copolymer was confirmed by neutrons scattering analysis. Gel mobility shift assays indicated that the presence of a PEG tail only slightly affected the DNA condensing properties of the LPEI. Moreover, in vitro gene transfection studies have shown that copolymer **9** has a significant transfection potential, in contrast to conjugates obtained by grafting PEG residues to the PEI. Taken together, we believe that our approach allowing for the synthesis of diblock copolymers containing a high molar mass PEI opens the way for the development of even more effective vectors.

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Supporting Information Available. Equations of the Kelen-Tüdos method. This material is available free of charge via the Internet at http://pubs.acs.org.

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