The Macrostopper Route: A New Synthesis Concept Leading Exclusively to Diblock Copolymers with Enhanced DNA Condensation Potential

Holger Petersen,[†,§] Alison L. Martin,[‡] Snjezana Stolnik,[‡] Clive J. Roberts,[‡] Martyn C. Davies,[‡] and Thomas Kissel*[,†]

Department of Pharmaceutics and Biopharmacy, Philipps-University of Marburg, Ketzerbach 63, D-35032 Marburg, Germany, and School of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

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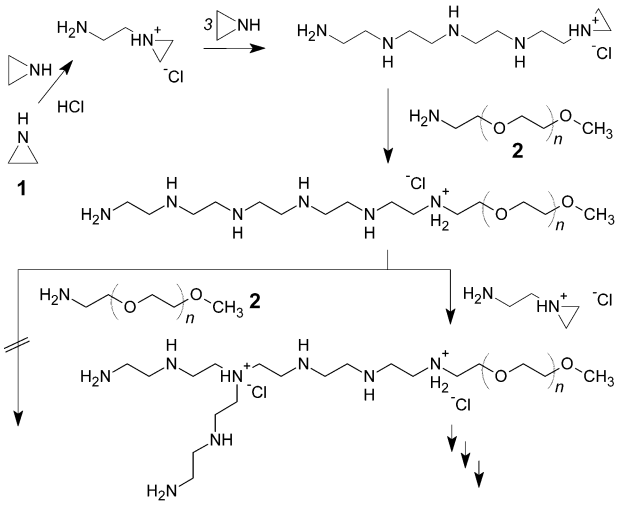
Introduction. Interpolyelectrolyte complexes (IPEC) of DNA and synthetic polycations have been proposed as a promising alternative to viral gene delivery systems.[1] However, the lack of stability of those complexes has led to the development of block copolymers composed of one cationic block for DNA condensation grafted with several nonionic hydrophilic blocks to enhance the complex solubility and shielding them against nonspecific interactions with biological components.[2] Prominent homopolymers for such copolymers are the cationic polyethylenimine (PEI), which is an effective gene transfection agent,[3] and poly(ethylene glycol) (PEG), a well-known shielding moiety.[4] Unfortunately, direct PEGylation of polycations such as PEI can diminish the DNA condensation properties.[5] To overcome this problem, we synthesized pure diblock copolymers and discovered that PEI-induced DNA condensation is not hampered when the polycation is grafted with only one PEG.

PEI is a hyperbranched polycation with many terminal amino groups. The synthesis of a pure diblock copolymer composed of branched PEI and linear PEG is not feasible via coupling of the homopolymers since in this case side products with more than one PEG per PEI macromolecule would also result.[6] The use of a PEG-macroinitiator for the ring-opening polymerization (ROP) of ethylenimine (EI) would also result in side products with higher degrees of PEGylation.[7] The only possibility to avoid multiple PEG grafting of PEI is to terminate the polymerization of EI by a PEG macromolecule. According to this function, we propose the term "macrostopper".

Experimental Section. *Diblock Copolymer Synthesis.* 1 mL of EI and 0.83 g of the monoamino-PEG were dissolved in a 25 mL flask containing 10 mL of water. The reaction was initiated using 50 μL of a 37% hydrochloric acid and stirred for 4 days at 25 °C. Finally, the solution was heated to 60 °C for 24 h. The solvent was removed under reduced pressure. The polymer was dissolved in ethanol and precipitated in diethyl ether, and the isolated polymer was dried in vacuo. PEG(5K)-*b*-PEI: 0.74 g, 40% yield. PEG(10K)-*b*-PEI: 0.82 g, 45% yield. PEG(20K)-*b*-PEI: 0.92 g, 50% yield. (The relatively poor yield is due to the poor precipitation of the copolymers in diethyl ether.)

Scheme 1. Synthesis of the Copolymers via the Macrostopper Route



Copolymer Characterization. SEC eluograms of the copolymers in 1% formic acid were detected by a refractive index detector RI-71 from Merck (Darmstadt, Germany) with MWs of the appropriate polymer fractions as determined by a multiple-angle laser light scattering detector from Wyatt Technologies (DAWN EOS, Santa Barbara, CA). Column: Suprema Max 3000 from Polymer Standard Service (Mainz, Germany). Flow rate: 1 mL/min. Temperature: 35 °C. MWs were determined via Zimm plots. Details were described previously.[8]

Copolymer/DNA Complex Characterization. AFM images of copolymer/pDNA complexes were imaged under 10 mM NaCl on a mica substrate. Images were conducted on a Dimension3000 scanning probe microscope from Digital Instruments (Santa Barbara, CA) with a Nanoscope IIIa controller. N/P ratio: 9. Plasmid: pBR322, 4363 bp, Sigma (St. Louis, MO). Details were described previously.[9]

Results and Discussion. The ROP of EI **1**, initiated by hydrochloric acid, was performed in aqueous solution containing the monomethyl-monoamino-PEG **2** as macrostopper (Scheme 1). It is well-known that low molecular weight (MW) amines terminate the polymerization reaction of EI.[10] Thus, we expected the same termination with high MW amines, such as monoamino-PEG. The nature of the EI polymerization mechanism ensures formation of diblock copolymers, exclusively. Once the propagating PEI chain is terminated by the PEG, the resulting copolymer can no longer react with a second PEG **2**. Only coupling of further oligoethylenimine with a reactive, alkylated three-membered ring to the PEI block is possible, leading to the formation of the hyperbranched structure of PEI. We performed this polymerization using a monoamino-PEG with MW of 5000 (for which we determined a MW of 7370 by light scattering (LS), Figure 1). The resulting copolymer exhibited a number-average MW (M_n) of 11 870, which is in line with the theoretically expected MW of 11 780 since the

† Philipps-University of Marburg.
‡ University of Nottingham.
§ Current address: Novartis Pharma AG, WSJ-340.755, CH-4002 Basel, Switzerland.
* To whom all correspondence should be addressed: Tel +49-6421-282-5881; Fax +49-6421-282-7016; e-mail kissel@mailer.uni-marburg.de.

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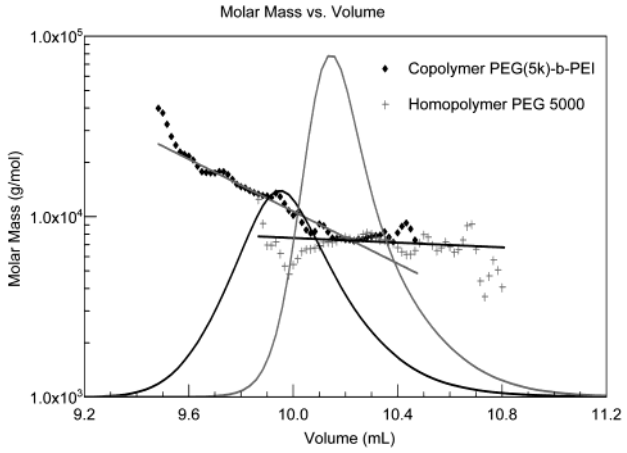

Figure 1. SEC eluograms in 1% formic acid as detected by a refractive index detector with MWs of the appropriate polymer fractions as determined by a multiple-angle laser LS detector.

M_n of PEI in a control experiment without macrostopper was found to be 4410.

The formation of the copolymers was also confirmed by ^{13}C NMR. The signals of the homopolymer end group PEG−O−CH_2−CH_2−NH_2 at 71.4 and 40.8 ppm were shifted to 70 ppm (overlapped by the PEG signal) and to 46 ppm (overlapped by one of the PEI signals) for the copolymer. All other signals appeared at the same chemical shift since we produced a copolymer without a linker group that would cause further shifts of signals. The absence of any ester or amide bonds for the linkage between the two polymer blocks yielded a copolymer which is extremely stable against hydrolysis. A ^{13}C-NNE-NMR experiment that allowed the integration of PEI's ethylene carbon signals by avoiding the nuclear Overhauser effect (NOE) demonstrated the unique degree of branching of the PEI block. While for commercially available PEIs the ratio of primary/secondary/ternary amino groups was found to be approximately 1/1/1,[11] the PEI block in this copolymer exhibited a more linear structure (ratio 1/2/1).[12] This lower degree of branching could be advantageous since linear PEI exhibited a higher activity in gene transfection experiments in vitro.[13]

While the macrostopper route perfectly functioned for PEG with MW of 5000, the feasibility of this route for higher PEG MWs seems to be limited. The ROP of EI in the presence of PEGs with MWs of 10 000 and 20 000 led to the formation polymers with M_n of 8000 and a weight-average MW (M_w) of 11 000. With a MW > 10 000 the terminal amino groups of PEGs seem to be no longer sufficiently accessible. This led to the formation of a blend consisting of unreacted PEG, PEI, and copolymer. With increasing MW of the macrostopper the amount of free homopolymer increased.

IPEC were prepared upon mixing aqueous solutions of PEG-*b*-PEI and plasmid DNA ($c = 40\ \mu$g/mL), in equal volumes in 10 mM NaCl, at pH 7.4 and at a PEI-amines/DNA-phosphate (N/P) ratio of 9. These self-assembling complexes were imaged by atomic force microscopy (AFM) under 10 mM NaCl solution. Uniformly spherical complexes with diameters of about 100 nm were observed for the PEG(5k)-*b*-PEI copolymer (Figure 2C). The compact appearance of the complexes formed with this diblock copolymer supports the impression of very efficient DNA condensation. This is in contrast to multigrafted PEGylated PEIs where poorly condensed DNA was observed (Figure 2A,B). PEG(10K)-*b*-PEI and

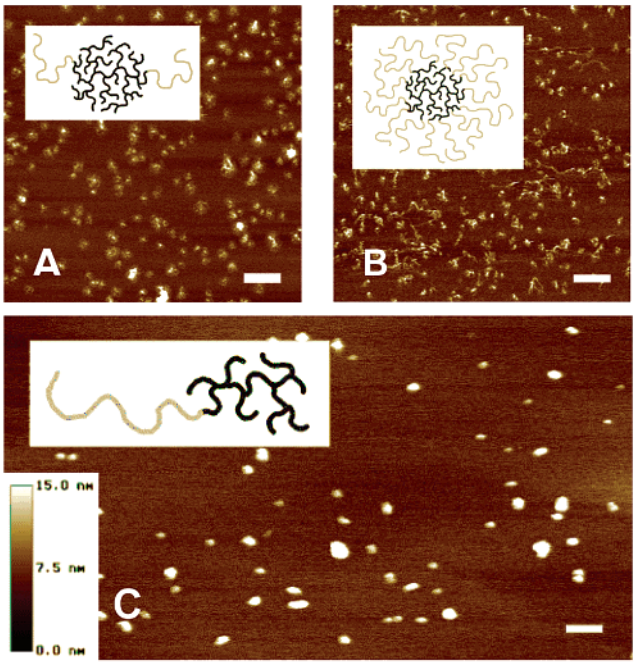

Figure 2. AFM images of copolymer/pDNA complexes at N/P = 9, 10 mM NaCl, pH 7.4. PEI 25 000 grafted with two (A) and 15 (B) PEG 5000 blocks on average. MonoPEGylated PEI: PEG(5k)-*b*-PEI (C). The scale bar is equivalent to 500 nm.

PEG(20K)-*b*-PEI also formed compact spherical complexes of about 100 nm diameter. These results were confirmed by dynamic LS (DLS) analysis demonstrating an enhanced DNA condensation of the diblock copolymers compared with the homopolymer PEI. Significantly smaller hydrodynamic diameters of about 100 ± 15 nm were found for all three copolymers, in comparison with complexes of the homopolymer PEI (325 ± 38 nm). The surface charge of the IPECs was estimated by ζ-potential measurements. The ζ-potential of the complexes was reduced from 24 ± 3 mV (no PEG) to 19 ± 3 mV by the PEG 5000 blocks and to 13 ± 4 mV by PEG 10 000. The stability and solubility of the complexes under critical conditions (neutral net charge and high ionic strength) were studied by DLS. Whereas PEI formed complexes aggregating to particles with diameters up to 800 nm within 20 min, the copolymers yielded stable complexes.

Conclusion. To summarize, our results have shown that the macrostopper concept led to diblock copolymers that enhance DNA condensation in comparison with homopolymer PEI and multi-PEGylated PEIs. Therefore, these PEG-*b*-PEI diblock copolymers are promising candidates for gene delivery, and their potential to transfect genes in vitro is currently investigated in our laboratories.

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Supporting Information Available: Scheme of the macroinitiator route, ^{13}C NMR spectra, MW data, elemental (C, H, N) analysis data, SEC eluograms, DLS/ζ-potential measurement data, and AFM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) The reaction of PEG blocks onto PEI in a stoichiometric 1:1 ratio is to a large extent a random process, which should lead to significant populations of PEI without any PEG blocks and with two or even more PEG blocks.

(7) Two PEG blocks that initiated the ROP of EI are able to react with each other during the propagation steps. The reactive site (the alkylated three-membered ring) of the one growing copolymer might react with the amines of PEI of the other copolymer.

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