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Engineering biodegradable micelles of polyethylenimine-based amphiphilic block copolymers for efficient DNA and siRNA delivery

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Abstract
Polycationic micelles have shown advantageous properties as nucleic acid delivery vectors both in vitro and in vivo. In contrast to polycationic micelles reported so far, we designed particles integrating a sufficient nucleic acid condensation capability by polycationic polyethylenimine (PEI) segments as well as only a mild cytotoxic behavior. The micelles composed of a hydrophobic oligoester core with glycolide units resulting in fast degradation after cellular internalization in combination with PEG moieties acting as shielding agents. By grafting branched 25 kDa polyethylenimine (PEI25) and poly(ethylene glycol) (PEG) on poly[(\varepsilon-caprolactone)-co-glycolide] (CG), amphiphilic PEI-CG-PEI and PEG-CG block copolymers were used to form a series of micelles via self-assembly of PEI-CG-PEI or co-assembly of both copolymers for DNA and siRNA delivery. This modular system enabled a
systematic investigation of different parameters and their synergetic effects as different functions were introduced. The polyplex formation and serum stability, cytotoxicity, and transfection activity could be tailored by changing the CG chain length in PEI-based copolymer, incorporating PEG-CG, and varying the N/P ratio. All micelle-based polyplex compositions showed high DNA transfection activity according to reporter gene-expression and an exceptionally high knockdown in siRNA delivery experiments. Remarkably, the GFP expression of >99% cells was successfully knocked down by micelle-mediated siRNA interference, resulting in a decrease of two orders of magnitude in fluorescence intensity. Incorporation of PEG-CG in the micelles reduced the PEI-related cytotoxicity, and markedly enhanced the serum stability of both DNA and siRNA polyplexes. Compared with homo-PEI25, these micelles showed several advantages including the lower toxicity, higher siRNA transfection efficiency and higher polyplex stability in the presence of serum. This study therefore provides an effective approach to tune the structure, property and function of polycationic micelles for efficient DNA and siRNA delivery, which could contribute to the design and development of novel non-viral transfection vectors with superb functionality.

**Keywords:** polyethylenimine, micelle, gene delivery, DNA, siRNA

1. **Introduction**

The transfer of nucleic acids with therapeutic functions into cells to modify defined cellular processes is a promising strategy for disease treatment [1,2]. There is a demand to apply different types of nucleic acids depending on the treatment strategy, including DNA [3], siRNA [4], miRNA [5], mRNA [6], and antisense oligonucleotide [7]. However, a series of extracellular and intracellular barriers must be conquered to achieve an efficient delivery of nucleic acids to the desired intracellular sites to fulfill their functions. For example, DNA delivered via systemic administration can be rapidly degraded by nucleases in the plasma [8]. The recognition and uptake of DNA complexes by the reticuloendothelial system (RES) might lead to a rapid clearance of DNA from the circulation [9]. DNA may be susceptible to nucleases when passing through the extracellular matrix (ECM) [10]. After reaching the target cells, DNA needs to overcome several barriers for their cellular internalization, endosomal escape, cytosolic transport and nuclear entry to finally arrive in the cell nucleus [11].
Although transit across the nuclear membrane is not necessary in case of siRNA and mRNA, their intrinsic properties including hydrophilic nature, negative charge and high molecular weight still limit their permeability across biological barriers [12].

By now, various delivery techniques and carriers have been developed to enhance the transfection efficiency of nucleic acids. Notably, polyethylenimine (PEI) has been most widely applied and studied due to its high effectiveness for condensing and delivering nucleic acids [11,13,14]. This complexation of PEI with nucleic acid materials could accelerate the process of polyplex endocytosis. Intracellularly, PEI with a branched structure might induce a so-called proton sponge effect, thereby triggering the rupture of endosomes and facilitating the endosomal escape of the delivered nucleic acid [15]. The branched PEI of 25 kDa (PEI25) has shown a superior transfection activity and has become a gold standard for gene therapy by polymeric carriers [16,17]. Similarly, high siRNA delivery capacity was reported for PEI25 compared to 22 kDa linear PEI and 800 Da branched PEI [14]. However, the relatively high toxicity of PEI, which has been attributed to both necrotic and apoptotic mechanisms resulting from cell membrane damage [18,19], is one of the major factors limiting its use, especially for in vivo applications. An effective strategy to reduce the toxicity of PEI has been demonstrated by covalent attachment of poly(ethylene glycol) (PEG) to PEI polymers [20,21]. As a nonionic hydrophilic polymer, PEG could shield the surface charge of polyplexes and reduce the intermolecular interactions by forming a hydration shell around the polyplexes, and hence decrease their toxicity. Importantly, PEGylation (i.e., PEG modification) has been shown to hinder the interaction of polyplexes with blood components. This feature is critical to enhance the serum stability of polyplexes, reduce the clearance by RES and prolong their blood circulation lifetime after intravenous administration [9,22–24].

In recent years, polymeric micelles have attracted interest due to their potential applications in nanomedicine [25–27]. They are usually formed by self-assembly of amphiphilic block- or graft-copolymers with a typical core-shell morphology. Compared to homo-polymers like PEI, polymeric micelles might offer several unique advantageous features for nucleic acid delivery such as the capacity to condense and protect the nucleic acid segment, while showing a higher colloidal stability, longer in vivo circulation time, improved cell association and internalization, enhanced transfection efficiency as well as lower toxicity [26,28]. Importantly, their physical and biological properties can be easily tuned by using multiple copolymers with
different shell-forming blocks to form co-assembled micellar structures [27,29,30]. Considering potentially strong effects of micelle composition on biological processes, an enhanced penetration of (intra-)cellular barriers may be achieved by incorporation of different functions, namely in the most important aspects (i) PEI as polycation for nucleic acid condensation, (ii) hydrophobic segments to enable the formation of a micellar structure, (iii) repetitive units that serve as weak links in the hydrophobic block for intracellular degradation, and (iv) mixed micelle concepts that allow to add additional features such as PEG to reduce cytotoxicity and increase serum stability via a shielding effect.

In this context, the aim of this study was to investigate the branched PEI-based micelles with tunable composition and structure as non-viral transfection vectors. Two amphiphilic block copolymers were utilized for micelle formation: (i) triblock PEI-CG-PEI as polycationic copolymer for nucleic acid condensation synthesized by grafting branched PEI25 on poly[(\(\varepsilon\)-caprolactone)-co-glycolide] (CG) with different molecular weights (6 kDa (CG6) and 10 kDa (CG10)); (ii) diblock copolymer PEG-CG for micelle PEGylation, which was prepared by grafting PEG (5 kDa) on CG (5 kDa). The series of micelles were prepared via self-assembly of PEI-CG-PEI or coassembly of both copolymers (1:1 wt%) in PBS (pH = 7.4), whereby a hydrophobic CG core providing hydrolytic degradable bonds and a hydrophilic PEI shell (or mixed shell with PEG segments to enable the shielding effect) can be formed (Scheme 1). The two PEI molecules conjugated at both ends of CG are supposed to provide high density of amino groups for nucleic acid condensation. Although the PEG-CG contains only one PEG molecule at one end of CG, the PEGylation degree could be easily controlled by varying the ratio of PEG-CG to PEI-CG-PEI. The glycolide units in the CG block (20 mol% for CG6 and CG10, 46 mol% for CG conjugated with PEG) were expected to promote the copolymer degradation. The higher amount of glycolide units in PEG-CG than in PEI-CG-PEI was expected to induce faster degradation of the CG block in PEG-CG in acidic pH of the early endosome and thereby eliminate the shielding effect from PEG after cellular internalization of the polyplexes. This might contribute to efficient transgene expression, as the wrapping of PEG chains on DNA could block the intracellular release of DNA and hence reduce the transfection efficiency [31,32]. The PEI-CG-PEI with different CG molecular weight might affect the size of initial micelles and consequently endow the micelles with different nucleic
acid loading capacity as well as transfection efficiency. The optionally incorporated PEG segments could form a PEG corona on the micelle surface, leading to a micellar structure with mixed hydrophilic shell. These micelles might have different condensation capacity for nucleic acids with respect to the polyplex size and zeta potential, both of which are critical for cellular binding of polyplexes and internalization via endocytosis [33–36]. Importantly, these PEG-containing micelles were expected to have reduced cytotoxicity and enhanced serum stability with high potential for in vivo applications. By using such a modular system, we were able to systematically investigate multiple parameters that are critical for micelle-mediated nucleic acid delivery including the chain length of hydrophobic segment, incorporation of shielding molecules, copolymer biodegradability and their synergetic effects on micelle characteristics, nucleic acid condensation, cytotoxicity, serum stability and transfection efficiency. In addition, as we used branched PEI25 for the hydrophilic segments of the cationic copolymers, we could compare these micelles with branched PEI25 gold standard to study the influence of micellar structure on transfection. Even more, we speculated that the transfection activity of the micelle-based polyplexes could be optimized by varying these parameters. Instructive knowledge could be gained from this modular system, with high potential for design and development of safe and efficient polymeric gene transfer agents for both in vitro and in vivo applications. Therefore, the prepared micelles were evaluated with respect to their condensation capacity, cytotoxicity, transfection efficiency for both DNA and siRNA as well as the stability of the polyplexes in the presence of serum.

2. Materials and methods

2.1. Synthesis of block copolymers and formation of polymeric micelles

The triblock PEI-CG-PEI copolymers were synthesized in a three step procedure. The hydrophobic CG oligoester was obtained by ring-opening polymerization of $\varepsilon$-caprolactone and diglycolide using octanediol as initiator. The resulting hydroxyl end groups were functionalized with succinic anhydride to create carboxylic acid moieties, which were subsequently modified with branched PEI25. The diblock copolymer PEG-CG was achieved by ring-opening polymerization of $\varepsilon$-caprolactone and diglycolide initiated by monohydroxy PEG-OH.

Polymeric micelles were prepared by dissolving PEI-CG-PEI copolymers (or mixture of PEI-CG-
PEI and PEG-CG) in DMSO and the resulting solution was added dropwise to the PBS solution to enable the formation of micelles by self-assembly. DMSO was removed by dialysis with PBS buffer. The sizes of micelles ranged between 19 ± 1 nm and 43 ± 2 nm as detected by Dynamic Light Scattering (DLS). The zeta potential was between 11.4 ± 0.3 mV and 17.8 ± 0.9 mV.

2.2. Amplification and purification of plasmid DNA
Luciferase plasmid (pcDNA3.1-Luc, Invitrogen, Carlsbad, CA, USA) was transformed into *Escherichia coli* DH5α strain and propagated in selective Luria-Bertani medium at 37 °C by shaking overnight at 200 rpm. The purification of amplified plasmid DNA was performed using the plasmid DNA purification kit (Macherey-Nagel, Düren, Germany). The purified plasmid DNA was dissolved in Tris-EDTA (TE) buffer and concentrated in a lyophilizer. The concentration and purity of plasmid were determined by loading the DNA onto the NanoQuant plate followed by measuring the ultraviolet (UV) absorbance at 260 and 280 nm with a microplate reader (Infinite 200 PRO®, Tecan Group Ltd., Mannedorf, Switzerland).

Scheme 1. (A) Formation of micelles by self-assembly of PEI-CG-PEI copolymer or co-assembly with PEG-CG as carriers for DNA and siRNA delivery. (B) Chemical structure of PEI-CG-PEI triblock and PEG-CG diblock copolymers.
2.3. Polymer/DNA polyplex preparation and characterization

Plasmid DNA was diluted with PBS to a concentration of 0.02 mg·mL⁻¹. Then, the equal volume of polymer solution (in PBS) was added dropwise into DNA solution, followed by a vortex for 30 s and incubation for 30 min at room temperature. The polymer/DNA ratio (N/P ratio), where ‘N’ is the molar amount of nitrogen from the primary amine of polymers and ‘P’ is the molar amount of phosphate from plasmid DNA, was calculated by taking into account that 1 μg DNA contains 3 nmol of phosphate and that 43 ng PEI (1 nmol of C₂H₅N repeat units) holds 0.25 nmol of primary amine nitrogen. The capability of polymers to condense plasmid DNA was studied by gel electrophoresis. Polyplex with various N/P ratios were mixed with glycerol (20% in PBS) and loaded onto the agarose gel (1.5%). Changes in the electrophoretic mobility of the samples were analysed by electrophoresis at room temperature in Tris-acetic acid-EDTA (TAE) buffer at 100 V. Then, the gel was stained with ethidium bromide solution and the DNA bands were visualized using an UV illuminator (ChemiDoc™ XRS+, Bio-Rad, Hercules, CA, USA). The polyplex size was determined by DLS using a ZetasizerNano from SZ instruments (Malvern Instruments, Herrenberg, Germany). The zeta potential was analysed with the same instrumentation. The measurements were performed at 25 °C using non-diluted samples in disposable cuvettes. The stability of polyplexes was determined by turbidity assays. Fetal bovine serum (FBS) was added into the prepared polyplexes to a final FBS concentration of 10%. Then the mixture was transferred into a 96-well tissue culture plate (TCP) (100 μL per well) and incubated at 37 °C. The aggregation in terms of turbidity increase was quantified by measuring the absorbance at 595 nm using a microplate reader (Infinite 200 PRO®, Tecan Group Ltd., Mannedorf, Switzerland).

2.4. Cytotoxicity assay

Human embryonic kidney 293 (HEK293; ATCC CRL-1573) cell line was cultured in a humidified incubator at 37 °C with 5% CO₂, in Dulbecco's minimum essential medium (DMEM) supplemented with fetal bovine serum (10%), streptomycin (100 μg·mL⁻¹) and penicillin G (100 IU·mL⁻¹). For cell viability assay, the cells were seeded into 96-well TCP with the number of 0.8 × 10⁴ cells per well. After 24 h of culture, the culture medium was replaced with fresh medium (150 μL per well), and the polyplexes with various N/P ratios as
well as naked DNA were added with a DNA dosage of 0.3 μg per well. In parallel, the commercial product Lipofectamine® 2000 (thermo Fisher Scientific, Schwerte, Germany) was used as a reference reagent following the given protocol. The Lipofectamine® 2000/DNA lipoplex was prepared by adding diluted DNA (0.02 mg·mL⁻¹ in serum free DMEM) to the equal volume of diluted Lipofectamine® 2000 (6 μL diluted with serum free DMEM to a final volume of 100 μL). Then, the lipoplex was incubated at room temperature for 5 min and added to cells with the same DNA dosage. After 48 h of incubation at 37 °C, 20 μL MTT (5 mg·mL⁻¹ in PBS) was added into each well. After another 4 h of incubation at 37 °C, the medium was removed and 150 μL DMSO was added into each well to dissolve the formed purple crystals. Absorbance was measured at a wavelength of 570 nm and a reference wavelength of 630 nm using a microplate reader (Infinite 200 PRO®, Tecan Group Ltd., Mannedorf, Switzerland). The results were expressed as the percentage of viability relative to the control cells, which were cultured without any treatment. Cell viability was calculated using the equation: Cell Viability (%) = \( \frac{(\text{OD}_{570} - \text{OD}_{630, \text{samples}})}{\text{OD}_{570} - \text{OD}_{630, \text{control}}} \) × 100%.

2.5. DNA transfection assay
For luciferase reporter gene transfections, HEK293 cells were seeded into 24-well TCP with a number of 4.0 × 10⁴ cells per well. After 24 h of culture, the culture medium was replaced with fresh medium (1 mL per well). The polymer/DNA polyplex, Lipofectamine® 2000/DNA lipoplex, as well as naked DNA were added with the DNA dosage of 2 μg per well. After 48 h of transfection, the culture medium was removed. The cells were washed twice by PBS and permeabilized by 250 μL cell lysis buffer (Promega, Madison, WI, USA). The luciferase level in cell extracts was measured using a Luciferase Assay Kit (Steady-Glo, Promega, Madison, WI, USA). The relative light units (RLU) were normalized against the amount of total protein in the cell extracts, which was measured using the BCA Protein Assay Kit (Pierce, Rockford IL, USA).

2.6. Polymer/siRNA polyplex preparation and siRNA delivery
The GFP siRNA (Stealth RNAi™ GFP Reporter Control, Thermo Fisher Scientific, Schwerte, Germany) and the scrambled control siRNA (Stealth RNAi™ siRNA Negative Control, Thermo Fisher Scientific, Schwerte, Germany) were diluted with PBS to the concentration
of 1 μM. Then, the equal volume of polymer solution (in PBS) was added dropwise into siRNA solution, followed by a vortex for 30 s and incubation for 30 min at room temperature. The polymer/siRNA ratio (N/P ratio) was calculated by taking into account that 25 pmol siRNA contains 1 nmol of phosphate moieties. The stability of prepared polymer/GFP siRNA polyplexes was determined by turbidity assay using the same protocol as that for polymer/DNA polyplexes. The commercial product Lipofectamine® 2000 (Thermo Fisher Scientific, Schwerte, Germany) was used as a reference reagent to evaluate the transfection efficiency, and the lipoplex was prepared with the ratio of 1 nmol siRNA/50 μL Lipofectamine® 2000 reagent according to the given protocol. The siRNA was diluted with serum free DMEM to the concentration of 1 μM and Lipofectamine® 2000 was diluted twenty times with serum free DMEM. Then, the diluted siRNA and the equal volume of Lipofectamine® 2000 solution was mixed and incubated at room temperature for 30 min.

GFP⁺-HeLa cells were derived from the HeLa cell line (ATCC; CCl-2), which were stably transfected with an expression unit for a destabilized enhanced green fluorescent protein (d2EGFP, Clontech, Palo Alto, USA). These GFP⁺-HeLa cells were used to quantitatively assess the efficacy of siRNA-mediated knockdown of the fluorescent reporter gene function upon delivery of the siRNA. Cells well cultured in DMEM medium supplemented with fetal bovine serum (10%), streptomycin (100 μg·mL⁻¹) and penicillin G (100 IU·mL⁻¹). For knockdown experiments, the cells were seeded into 24-well TCP with the number of 5.0 × 10⁴ cells per well. After 24 h of culture, the medium was replaced with fresh medium (500 μL per well) and the cells were transfected with polyplexes, lipoplex as well as naked siRNA. For each well, 50 pmol siRNA was used. 48 h post-transfection, the cells were observed with a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany). The GFP silencing efficiency was quantified using a flow cytometer (MACSQuant®, Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, the culture medium was removed and the cells were washed three times with PBS. Then, cells were harvested by trypsin-EDTA and washed twice with PBS. For each sample, at least 1.0 × 10⁴ cells were counted. Data were analysed with FlowJo software (Tree Star Inc., Ashland OR, USA).

2.7. Statistics
Statistical analysis was performed using the two-tailed independent-samples t-test, and a
significance level (Sig.) < 0.05 was considered to be statistically significant. Data are presented as mean ± standard deviation.

3. Results and discussion

3.1. Characterization of polymer/DNA polyplexes

The complexation of DNA by polymers was studied via a gel electrophoresis assay including PEI25 for comparison (Fig. 1). As expected, the capacity of polymers to reduce and ultimately prevent DNA migration increased with the increase of N/P ratio. However, a similar DNA immobilization was observed in all of the tested polymers, whereby the DNA was completely retarded at N/P ratio 1. The DNA retardation of PEI-based triblock copolymers was not altered by the difference of CG chain length as well as the involvement of PEG-CG cooperative polymer. The results demonstrate that a rather small amount of micellar carriers is sufficient to provide complete complexation, likely due to the high avidity arising from the micellar structure.

The particle size and zeta potential of the prepared polymer/DNA polyplexes at various N/P ratios was further investigated (Fig. 2). With the increase of N/P ratio, the polyplexes exhibited a trend towards decreasing particle size and increasing zeta potential, which was attributed to the gradually enhanced DNA condensing effect by increasing the amount of cationic polymers. However, from N/P ratio 4 to 16, the zeta potential remained relatively constant. This result is in consistence with the observations by other groups as well as the result of our previous work using cationic polymers as gene carriers [3,37–39]. It has been demonstrated that at high N/P ratios, a large amount of PEI was free in solution [40,41]. Therefore, in our system, there might be an excess of non-complexed micelles in the polyplex solution at N/P ratios higher than 4, resulting in the observed constant zeta potential. Notably, this plateau of zeta potential showed values similar to that of pure micelles, suggesting that free micelles dominated the determined zeta potential or there might be a structural organization of plasmid DNA and excess micelles, whereby the negative charge of the DNA was well shielded. It has been reported that the free cationic polymers in solution could contribute to efficient gene expression [41], indicating that also the bare micelles might play an important role in micelle mediated gene delivery.
Fig. 1. Agarose gel electrophoresis of polymer/DNA polyplexes prepared using PEI25 and different micelles. Lane 1, plasmid DNA only used as control; lanes 2–11, polyplexes at various N/P ratios from 0.25 to 2.5.

Fig. 2. Characterization of polyplex formation at various N/P ratios using PEI25 and different micelles. (A) Particle size, (B) zeta potential (n = 3).
Fig. 3. Cytotoxicity and transfection efficiency of naked DNA, Lipofectamine® 2000/DNA lipoplex and polymer/DNA polyplexes at different N/P ratios. (A) Cytotoxicity determined by MTT assay was expressed as the percentage of viable HEK293 cells compared to the untreated control cells ($n = 8$; *Sig < 0.05 as compared to PEI25 at the same N/P ratio; #Sig < 0.05 for cationic copolymers with PEG-CG vs corresponding copolymers without PEG-CG at the same N/P ratio). (B) The DNA transfection efficiency was quantified by delivering the luciferase plasmid to HEK293 cells and measuring the transgene expression level 48 h after transfection ($n = 6$; #Sig < 0.05 for cationic copolymers with PEG-CG vs corresponding copolymers without PEG-CG at the same N/P ratio).

Incorporation of PEG-CG during micelle formation could strongly affect the micelle-DNA interactions, as both particle size and zeta potential were altered at the presence of PEG-CG. It has been suggested that the PEG corona could prevent the aggregation of micelles or micelle-based nanocomposites due to a steric repulsion between the PEG chains [42–45]. This may explain that sizes of polyplex particles containing PEG-CG were smaller than those without PEG-CG for most of the N/P ratios (Fig. 2A), which was likely due to a lower number of micelles aggregating in PEG-CG containing polyplexes. This observation is in consistence with the result reported by Lin et al., where a chitosan-based polymer conjugated with PEG ($M_w = 5$...
kDa) resulted in much smaller polymer/DNA polypelex size than the corresponding polymer without PEG [46]. In addition, an influence of PEG-CG on the zeta potential was observed at most of the N/P ratios, i.e., the zeta potential was decreased by around 50% (Fig. 2B), which could be attributed to the shielding effect of PEG on the surface charge [23]. Taken together, these results indicate a cooperative synergistic effect of different amphiphilic copolymers as well as the polymer/DNA ratio on polymer/DNA complexation, which offers the opportunity to tune the properties and function of the polypelexes by varying different parameters.

3.2. Cytotoxicity and DNA transfection

The cytotoxicity and transfection efficiency were quantified to evaluate the prepared micelles as DNA delivery vectors (Fig. 3). The MTT assay showed a decrease of cell viability with the increase of the N/P ratio for all of the tested polymers (Fig. 3A), which could be attributed to cell death by the charge-induced cell membrane damage at high amounts of cationic polymers [18,19,47]. The addition of PEG-CG to PEI-CG10-PEI remarkably enhanced the cell viability at the N/P ratios ranging from 2 to 12, suggesting the shielding effect of PEG on the surface charge of polypelexes [20,21]. However, such an effect was not significant for PEI-CG6-PEI copolymer at most of the N/P ratios. This might be attributed to the weaker PEG shielding effect in the PEI-CG6-PEI/PEG-CG polypelexes. Compared to PEI-CG10-PEI, the shorter CG chain in PEI-CG6-PEI might result in a smaller hydrophobic core, which would lower the amount of PEG-CG copolymer incorporated into the micelle structure. This speculation was supported by the zeta potential result (Fig. 2B), as a higher zeta potential was observed in the PEI-CG6-PEI/PEG-CG polypelex compared to the PEI-CG10-PEI/PEG-CG polypelex at N/P ratios from 4 to 16. Further studies will be necessary to clarify the influence of the length of hydrophobic chains on the co-assembly of micelles and their DNA complexation capacity. Notably, compared to homo-PEI25, the cytotoxicity of polypelexes prepared using PEI-CG10-PEI/PEG-CG micelles was significantly lower, suggesting a general strategy of using such polypelexes to improve the viability of transfected cells.

The results of luciferase assay proved the capability of all prepared polypelexes to deliver DNA into the cells resulting in an efficient transgene expression (Fig. 3B). The highest transfection efficiency was observed at N/P ratio 4 for PEI25, N/P 8 for PEI-CG10-PEI, N/P 8 for PEI-CG10-PEI/PEG-CG, N/P 8 for PEI-CG6-PEI and N/P 12 for PEI-CG6-PEI/PEG-CG. Statistic
analysis showed that for the highest transfection efficiency, no significant difference between PEI25 and the four micelle systems, i.e., the latter were similarly effective but showed a higher cell viability compared to pure PEI. In addition, the polyplexes prepared using PEI-CG10-PEI, PEI-CG10-PEI/PEG-CG and PEI-CG6-PEI at N/P ratio 8 exhibited a similar transfection efficiency compared to the Lipofectamine® 2000/DNA lipoplex. These results underline the potential of the PEI-based block copolymer micelles as efficient gene carriers. The addition of PEG-CG remarkably increased the transgene expression at higher N/P ratios (20 and 24). Given the fact that the cells have a comparable viability at these N/P ratios, this was not due to a difference in cytotoxicity. There might be other mechanisms through which one or more transfection barriers were overcome by the presence of PEG-CG. Further studies focusing on the intracellular trafficking of the polyplexes may be helpful to clarify this issue.

In addition, we investigated whether the incorporation of glycolide could enhance the DNA transfection efficiency. By comparing the transfection efficiency of the glycolide containing copolymers with those without glycolide, namely PEI-C-PEI and PEG-C (C: poly(ε-caprolactone)), we did not observe dramatic differences between the copolymers with and without glycolide (data not shown). This might be because glycolide containing blocks were not degraded in the time period of the experiment (48 h), as the degradation of glycolide in PEI-CG-PEI could be only observed after N 2 days in the acidic environment of early endosome (pH 5.5) (data not shown). Therefore, the effect of CG degradation on transfection might be observed by prolonging the transfection time or further increasing the amount of glycolide in the copolymer.

3.3. siRNA transfection

The delivery potential of the prepared polyplexes for siRNA was investigated by assessing the efficacy of siRNA mediated knockdown of the GFP reporter gene using the GFP^+-HeLa cells (Figs. 4 and 5). The commercial product Lipofectamine® 2000, a highly efficient transfection reagent that has been widely used for DNA and siRNA delivery [48], was used as reference reagent. A low knockdown efficiency was observed in the cells transfected by lipoplex and polyplexes prepared using scrambled siRNA, indicating a weak sequence independent silencing effect. Efficient knockdown of GFP expression could be achieved by all of the tested polymers according to the results of flow cytometry analysis (Fig. 4) and fluorescence microscopy (Fig.
Remarkably, a decrease of two orders of magnitude in GFP fluorescence intensity was observed at higher N/P ratios (Fig. 4B). Compared to Lipofectamine® 2000, a substantially enhanced efficiency could be noted. At a N/P ratio 8, silencing was similar for PEI-CG6-PEI/PEG-CG and Lipofectamine® 2000, but more efficient for the other polyplexes. For instance, polyplexes prepared using PEI-CG10-PEI or PEI-CG6-PEI micelles resulted in only 0.6% and 1.2% GFP+ cells at N/P ratio 8, which was also significantly more efficient than the value achieved by PEI25 (1.8%).

Fig. 4. The GFP siRNA transfection efficiency in GFP+ HeLa cells of naked siRNA, Lipofectamine® 2000/siRNA lipoplex and polymer/siRNA polyplexes at different N/P ratios. The lipoplex and polyplexes prepared using scrambled siRNA were used as control. The percentage of GFP+ cells relative to total cells (A) and mean fluorescence intensity (MFI) (B) were quantified by flow cytometry 48 h after transfection (n = 3; *Sig < 0.05 as compared to
PEI25 at the same N/P ratio; *Sig < 0.05 for cationic copolymers with PEG-CG vs corresponding copolymers without PEG-CG at the same N/P ratio). (C) Representative histograms of flow cytometry analysis showed the GFP silencing efficiency of the cells transfected by different reagents.

Fig. 5. Representative fluorescence images of the GFP⁺-HeLa cells transfected with naked siRNA, Lipofectamine® 2000/siRNA lipoplex and polymer/siRNA polyplexes at various N/P ratios. The lipoplex and polyplexes prepared using scrambled siRNA were used as controls. The images were taken 48 h after transfection using a confocal laser scanning microscope.
The N/P ratio seemed to be a critical factor influencing the silencing efficiency, whereby an increased efficiency was observed with the increase of N/P ratio. At N/P ratio 4, PEI-CG10-PEI showed the highest silencing efficiency of the different polyplexes with a remaining 25% of GFP+ cells, while at N/P ratio 16 less than 1% of the cells were still GFP positive (Fig. 4A). Both the chain length of CG in PEI-based copolymer and the incorporation of PEG-CG could dramatically influence the silencing efficiency. At N/P ratio 4 and 8, the PEI-CG10-PEI polyplexes containing the longer CG chain was much more efficient than those from PEI-CG6-PEI, which has the shorter CG chain. This observation might be explained by two aspects. On the one hand, the size of initial micelles formed by PEI-CG10-PEI was around two times as large as the size of the micelles formed by PEI-CG6-PEI (∼40 nm vs ∼20 nm, data not shown). Considering that siRNA is approximately 7.5 nm in length and 2 nm in diameter [49], this size difference might endow the larger PEI-CG10-PEI micelles with a higher siRNA carrying capacity than the smaller PEI-CG6-PEI micelles, and hence lead to a higher degree of siRNA internalization. On the other hand, the smaller PEI-CG6-PEI micelles with a higher surface curvature might be able to expose more of their charged moieties to improve the siRNA binding. Further, considering the branched structure of PEI on the outer layer of micelles, the higher surface curvature might also facilitate the penetration of siRNA into the PEI shell allowing for stronger interaction. These effects might result in difficulties for siRNA dissociation from the polyplexes within the cells, thereby reducing the siRNA silencing efficiency. In addition, we found that the micelles without PEG-CG could lead to a higher silencing degree than those containing PEG-CG. This could be attributed to the lower endocytosis rate of micelles containing PEG, since PEGylation could reduce the cellular interaction and uptake of nanoparticles [50,51].

Taken together, these results showed that the polymeric micelles could serve as efficient delivery vectors for siRNA mediated gene knockdown. In a recent study reported by Lu et al., the co-delivery of DNA and siRNA via arginine-rich PEI-based polyplexes could significantly enhance gene transfection levels of both nucleic acids compared to that of either DNA or siRNA alone [52]. Following this research direction for gene transfer/therapy, the micellar carriers may be interesting for co-delivery of DNA and siRNA using synergetic effects to modulate cell behavior and function.
3.4. Polyplex stability

In this study, the PEG-CG was used together with PEI-based copolymers to form the micelles via co-assembly. One might expect that the polyplexes prepared from such mixed micelles have better serum stability than those prepared from micelles without PEG-CG and from homo-PEI25. Therefore, the polyplex stability was evaluated via a turbidity assay [53,54]. The polyplexes were incubated with 10% FBS and the solution turbidity was recorded up to 24 h (Fig. 6). The result showed that polyplexes containing PEG-CG were very stable in serum conditions. During the incubation, there were almost no increase of turbidity for PEG containing siRNA polyplexes and only a slightly increased turbidity for PEG containing DNA polyplexes at high N/P ratio, suggesting that the involvement of PEG is an effective strategy to improve the stability of the polyplexes. In contrast, both DNA and siRNA polyplexes prepared using PEI25 presented much lower serum stability, showing dramatically increased turbidity after 12 h of incubation.

Interestingly, a different behavior was observed for the DNA and siRNA polyplexes prepared using PEI-CG-PEI copolymers without PEG-CG. The DNA polyplexes were more stable in the presence of serum at lower N/P ratio, whereas the siRNA polyplexes were more stable at higher N/P ratios. At N/P ratio 12, the PEI-CG10-PEI/siRNA polyplexes were even as stable as those containing PEG-CG. Apparently, the different behavior on serum stability between the DNA and siRNA polyplexes was caused by the distinctive chemical and structural features of plasmid DNA and siRNA. Circular, supercoiled plasmid DNA has a much larger size (usually several kilobase pairs) than linear, relaxed siRNA (20–25 base pairs). In addition, due to the different conformation of DNA (B-form helixes) and RNA (A-form helixes), DNA has distinctive structural and physical properties in comparison to RNA, such as higher helical twist per base pair, larger distance between base pair, smaller helix diameter and lower rigidity [52,55]. Hence, when complexed by polycationic micelles, DNA and siRNA may show different behaviors in interacting with cationic polymers, which might contribute to the measured differences in serum stability. Further studies focusing on the structure and properties of DNA and siRNA polyplexes might reveal the exact interaction mechanism, which was outside the scope of this study.
Fig. 6. Colloidal stability of polymer/DNA and polymer/siRNA polyplexes formed at N/P ratio 4 and 12. PBS and the nucleic acid solution with the corresponding concentration to the polyplex solution were used as controls. The solution turbidity was determined at different time points by measuring the absorbance at 595 nm (n = 3; *Sig < 0.05 as compared to the initial absorbance at 0 h; #Sig < 0.05 for cationic copolymers with PEG-CG vs corresponding copolymers without PEG-CG at the same N/P ratio).

Conclusion
In summary, it was shown that the polycationic micelles providing different functions, e.g. condensation efficiency of PEI, glycolide induced degradability in combination with reduction of cytotoxicity and enhancement of serum stability as result of a shielding effect by PEG, were designed via self-assembly of PEI-CG-PEI or co-assembly with PEG-CG. This modular system was efficient for DNA and siRNA delivery. Our result demonstrated that the formation of micelle/nucleic acid polyplexes as well as their properties and transfection activity were controlled by multiple parameters, including the length of the hydrophobic part in PEI-CG-PEI, the incorporation of PEG-CG in mixed micelles, the polymer/nucleic acid ratio and the type of nucleic acid. By adjusting these parameters, the physical properties and delivery functions of the micelles for optimal transfection can be tailored. When compared to homo-PEI25, a lower cytotoxicity, comparable DNA transfection efficiency, higher siRNA transfection efficiency and higher serum stability of the polyplexes could be achieved by the prepared micelles. Further
studies may reveal if these carriers may also be suited for e.g. siRNA/DNA co-delivery, tissue penetration such as in the skin, or vaccination purposes.

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References
[33] S.L. Zhang, J. Li, G. Lykotrafitis, G. Bao, S. Suresh, Size-dependent endocytosis of


M.P. Xiong, M.L. Forrest, G. Ton, A. Zhao, N.M. Davies, G.S. Kwon, Poly(aspartate-g-PEI800), a polyethylenimine analogue of low toxicity and high transfection efficiency for gene delivery, Biomaterials 28 (2007) 4889–4900.


C. Wischke, M. Behl, A. Lendlein, Drug-releasing shape-memory polymers - the role of morphology, processing effects, and matrix degradation, Expert Opin. Drug Deliv. 10 (2013)