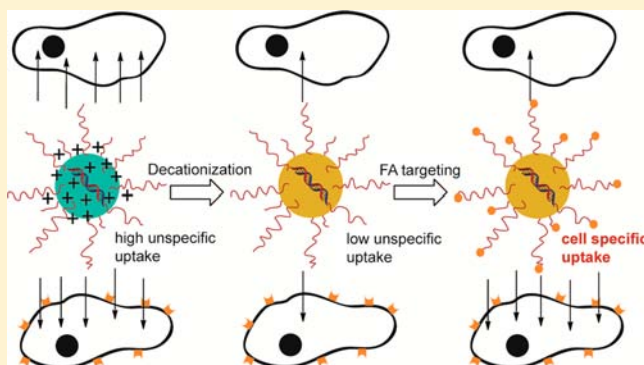


Targeted Decationized Polyplexes for Cell Specific Gene Delivery

Luís Novo, Enrico Mastrobattista, Cornelus F. van Nostrum, and Wim E. Hennink*

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, The Netherlands

ABSTRACT: Decationized polyplexes have previously shown unique features, especially regarding their excellent cytocompatibility and very low degree of nonspecific cellular uptake. In the present study, targeted disulfide cross-linked decationized polyplexes were composed of a core of disulfide cross-linked poly(hydroxypropyl methacrylamide) (pHPMA) stably entrapping plasmid DNA (pDNA) and a shell of poly(ethylene glycol) (PEG) decorated with folate molecules. Folate was used as targeting ligand because of its high binding affinity to its receptor, which is overexpressed in many tumors. Studies using folate receptor overexpressing cell lines (HeLa and OVCAR-3) showed significantly higher cell uptake for the folate-targeted decationized polyplexes, when compared to their nontargeted counterparts. On the contrary, for a nonexpressing folate receptor cell line (A549) similar uptake was observed for both targeted and nontargeted decationized polyplexes. Transfection studies using OVCAR-3 cells showed higher transfection efficiency for folate-targeted polyplexes, because of improved cellular uptake. Simultaneously, introduction of targeting moiety on polyplexes did not affect their good cytocompatibility. The results reported in this paper demonstrate that coupling of folate to decationized polyplexes generates a potential system for targeted gene delivery.



INTRODUCTION

Gene therapy is a highly promising modality for the treatment of many disorders with a genetic basis, including cancer.¹ The applicability of gene therapy relies on the emergence of gene delivery vectors with *in vivo* safety and effectiveness. Serious disadvantages are however associated with viral vectors when applied in patients, including insertional mutagenesis and immunogenicity.^{1,2} Therefore, nonviral gene delivery systems based on, e.g., cationic polymers, peptides, or lipids have been developed and investigated as a safer alternatives for viral vectors.^{3–9}

Cationic polymers are of particular interest since they can easily interact with the negatively charged DNA to form nanosized particles (polyplexes). However, the cationic surface charge of such systems leads to toxicity both *in vivo*^{10,11} and in cell cultures.¹² A major challenge of nonviral vectors is therefore the development of systems suitable for systemic applications. In order to target their site of action (e.g., a tumor), the polyplexes should first possess long circulation and stability to reach the target tissue and extravasate in the tissue leaky vasculature, referred to as the enhanced permeation and retention (EPR) effect.^{13,14} Only after sufficient accumulation can internalization of the vectors occur.¹⁵ Often, polycation based systems, even when shielded with hydrophilic polymers such as PEG, lead to insufficient improvements on the circulation times and tumor accumulation.^{16–18} After interaction with the target cells, polyplexes should induce efficient transgene expression and, at the same time, reduce the risk of interference with cell homeostasis to a minimum level.

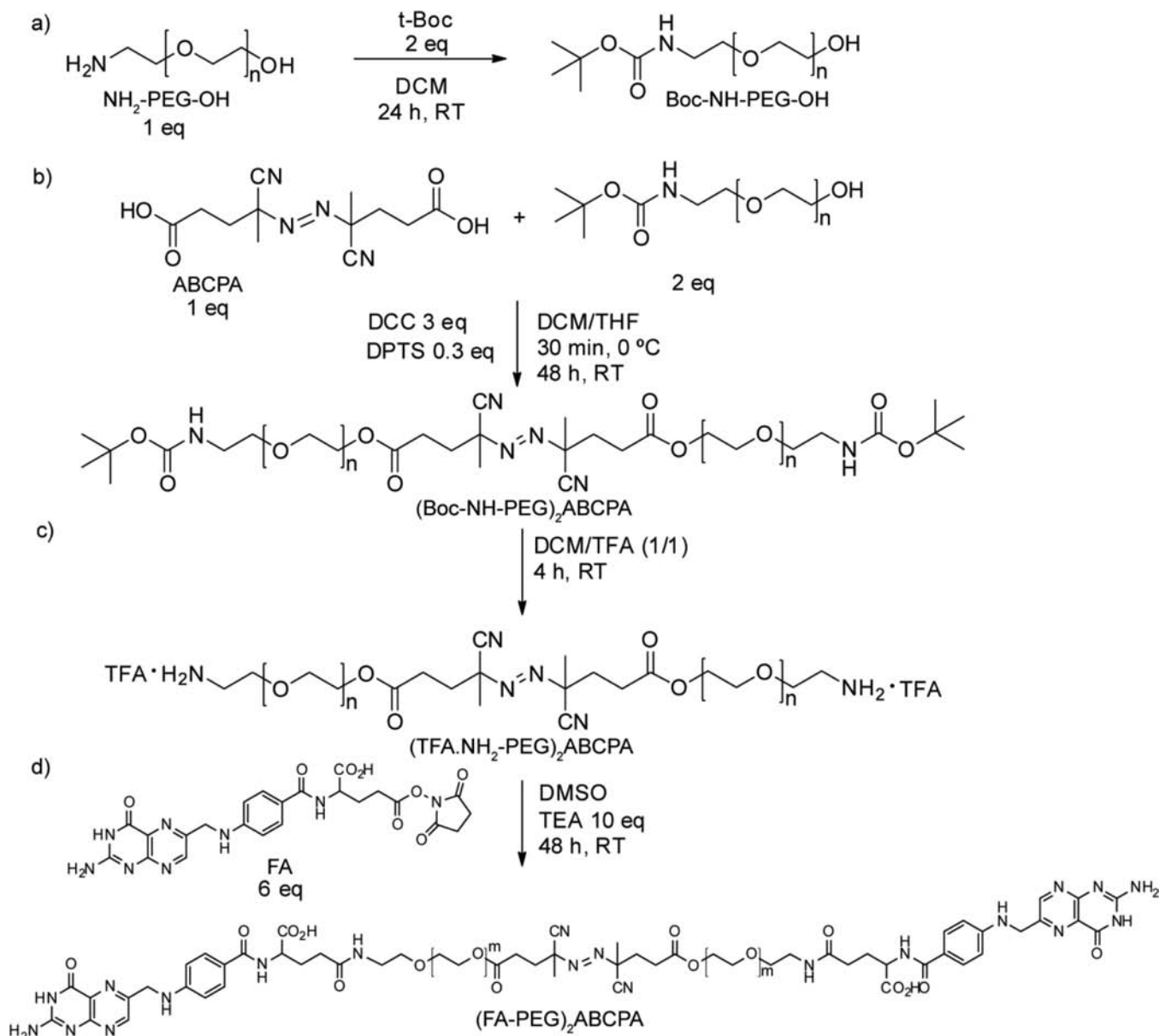
In a previous paper we reported on decationized polyplexes¹⁹ that, unlike conventional polymeric gene delivery systems, are based on noncharged and hydrophilic polymers. Structurally, the polyplexes possess a core of disulfide cross-linked pHPMA, surrounded by a PEG shell. Cationic charges are present on the polymers during complex formation with DNA, but are subsequently removed by hydrolysis leaving a hydrophilic disulfide cross-linked polyplex. These decationized polyplexes show a high and stable DNA loading, based exclusively on physical entrapment in the disulfide cross-linked core, and excellent cytocompatibility. We further demonstrated that pDNA is released from the polyplexes triggered by intracellular reducing environment that results in cleavage of disulfide cross-links.^{20,21} Importantly, the decationized polyplexes showed a low degree of nonspecific uptake, which on one hand significantly reduced their efficiency when compared to their cationic counterparts, but on the other hand is a highly favorable property for targeted therapy upon local or intravenous administration. Incorporation of a targeting ligand on the decationized polyplexes could lead not only to significantly increased cellular binding and internalization, required for efficient transfection, but also to create a gene delivery system with high specificity when compared to conventional polyplexes.

Folic acid (FA) selectively binds with high affinity to its receptor, the folate receptor (FR), which is overexpressed in

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Scheme 1. Synthesis Route of (FA-PEG)₂-ABCPA


many tumor types including metastatic forms, while having limited expression in normal tissues.²² Due to its particular anatomic distribution, expressed in apical plasma membrane of the polarized cells, FR is normally inaccessible via the bloodstream in normal tissues; however, loss of epithelial cell polarity, observed in tumors, makes the FR accessible from the circulation.^{23,24} Drug delivery systems conjugated to FA bind to FR on the cell surface and are subsequently internalized via receptor mediated endocytosis. Accordingly, folic acid is a suitable targeting ligand for tumor-specific targeted drug and gene delivery upon systemic administration.^{22,25,26}

In this study, we exploited the advantageous property of the very low degree of nonspecific cellular uptake of decationized polyplexes together with the folic acid targeting ability to design a polymeric gene delivery vector with high targeting specificity for FA overexpressing cells.

RESULTS AND DISCUSSION

Synthesis of pHDP-PEG with Terminal Folate Moiety.

To obtain prepare folate targeted decationized polyplexes, a protocol to synthesize a folate-PEG (M_w 5000 Da) bifunctionalized azo macronitiator ((FA-PEG)₂-ABCPA) was developed, based on the synthesis of mPEG₂-ABCPA previously described by Neradovic et al.²⁹ The rationale behind this route is that the (FA-PEG)₂-ABCPA will initiate free radical polymerization to form block copolymers with a folate functionality at the distal PEG end (Scheme 1). Consequently, the folic acid can be coupled to the macroinitiator, to allow a straightforward approach to obtain folic acid functionalized polymers that can be used for polyplex preparation, without the need of postmodification procedures.

The synthesis of (FA-PEG)₂-ABCPA (Scheme 1) starts with Boc protection of the primary amine (a) of the commercially available NH₂-PEG-OH. ¹H NMR analysis showed quantitative protection (¹H NMR (CDCl₃) δ (ppm) 1.4 (9H, Boc)) and primary amines were not detected using the TNBSA assay.

Next, Boc-NH-PEG-OH was coupled to ABCPA via DCC coupling (b). GPC analysis of the product (Figure 1) showed a

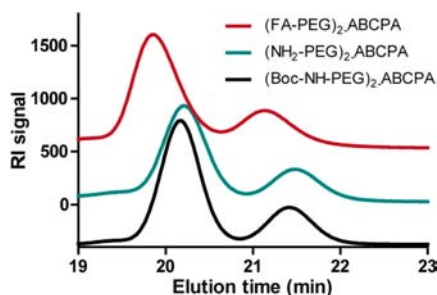


Figure 1. GPC chromatograms (RI signal) of (Boc-NH-PEG)₂-ABCPA, (NH₂-PEG)₂-ABCPA, and (FA-PEG)₂-ABCPA.

bimodal distribution, corresponding to a mixture of PEG bifunctionalized ABCPA (10 kDa) and unreacted Boc-PEG-OH or PEG monofunctionalized ABCPA (5 kDa). The ratio between 10 kDa and 5 kDa products was 77%/23%. The third step of the reaction scheme was the Boc deprotection with TFA, generating the TFA salt form of (NH₂-PEG)₂-ABCPA (c). ¹H NMR analysis showed the complete disappearance of the Boc signal at δ1.4 ppm and the TNBSA assay showed that the mol/mol ratio of primary amines to PEG chains was close to 1. Additionally, the GPC chromatogram showed that the ratio between 10 kDa and 5 kDa coproducts was retained. The last step of the synthesis was the reaction between NHS activated folic acid (FA-NHS) and the deprotected (TFA-NH₂-PEG)₂-ABCPA (d). The reaction was done under anhydrous conditions to avoid hydrolysis of FA-NHS and using a molar excess of FA-NHS to NH₂ groups from the macroinitiator to limit possible aminolysis of the ester bond that links the PEG chain and ABCPA.

To determine the coupling efficiency of folic acid to NH₂ groups in the macroinitiator, ¹H NMR was used to compare the integral values of the specific signal of folic acid with the integral values of the specific signal of PEG (Figure 2), which

showed that approximately 1 mol of folic acid was coupled to 1 mol of PEG chains. The GPC chromatogram of the (FA-PEG)₂-ABCPA shows again that the ratio of 10 kDa and 5 kDa coproducts was retained and that the excess of FA-NHS was removed by dialysis (Figure 3). The ¹H NMR and GPC results

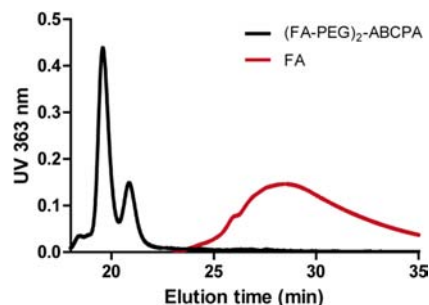


Figure 3. GPC chromatograms (UV signal at 363 nm) of (FA-PEG)₂-ABCPA and free folic acid (FA).

demonstrate that the applied synthetic route results lead to the formation of the aimed product, namely, PEG bifunctionalized macroinitiator with coupled folic acid moiety.

Free radical polymerization of HPMA-DMAE with PDTEMA using (FA-PEG)₂-ABCPA as macroinitiator resulted in the formation of pHDP-PEG-FA. A second polymer was prepared using the (Boc-PEG)₂-ABCPA, which was used to prepare nontargeted polyplexes (pHDP-PEG) (Scheme 3). The yield of both polymerizations was close to 50%, which suggests that the incorporation of folic acid molecule in the PEG₂-ABCPA macroinitiator did not affect the copolymerization reaction.

The cationic HPMA-DMAE monomer was incorporated in the copolymer to allow pDNA complexation by electrostatic interactions. Hydrolysis of the DMAE cationic groups³⁹ after interchain disulfide cross-linking yielded decationized polyplexes based on the hydrophilic PEG and pHPPMA polymers. The PDTEMA monomer, also a methacryamide monomer, was chosen because it contains a PDS functionality, which allows

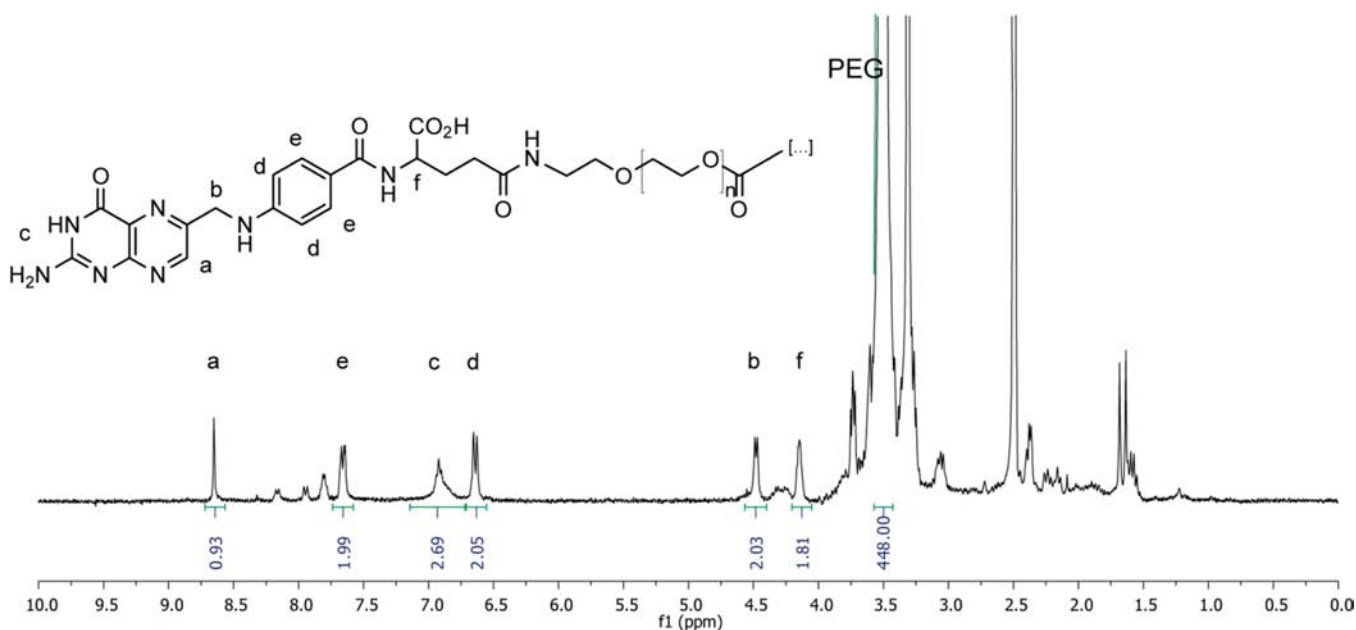
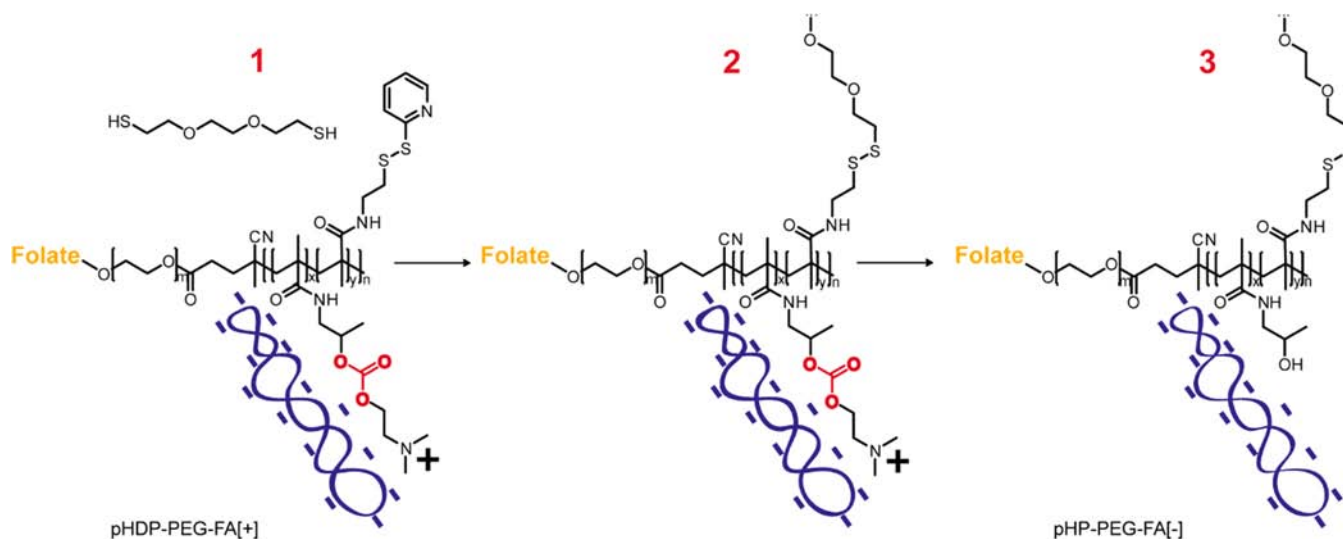


Figure 2. ¹H NMR spectrum of (FA-PEG)₂-ABCPA in DMSO.

Table 1. Overview of the Characteristics of the Synthesized pHDP-PEG-FA and pHDP-PEG as Determined by GPC, ^1H NMR, and UV Spectroscopy

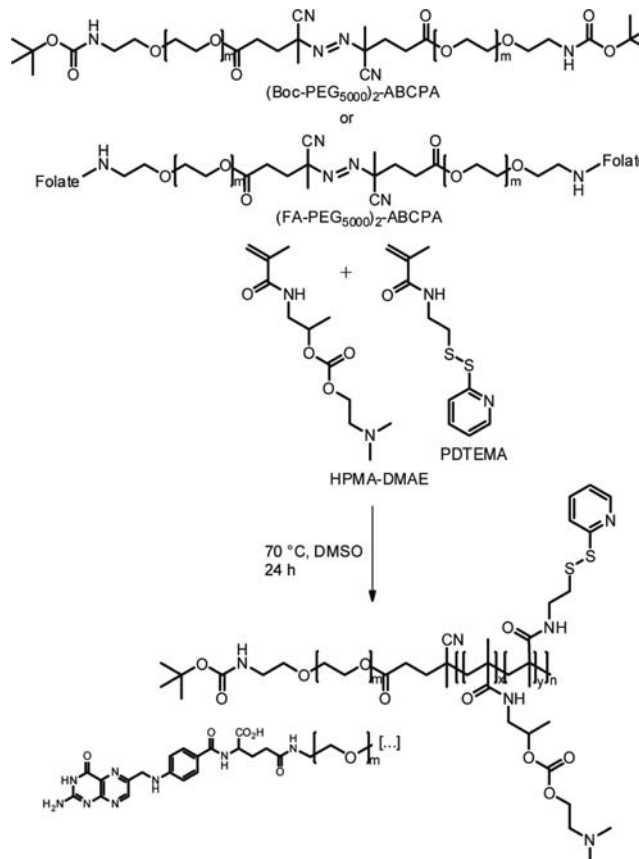
polymer	GPC			NMR			UV
	M_n (kDa)	PDI	dn/dc	M_n (kDa)	feed _{HPMA-DMAE/PDTEMA}	polymer _{HPMA-DMAE/PDTEMA}	
pHDP-PEG-FA	116.6	1.4	0.18	43.3	1/0.2	1/0.16	295 ± 9
pHDP-PEG	119.9	1.5	0.17	43.9	1/0.2	1/0.15	305 ± 2

Scheme 2. Route for the Preparation of Interchain Disulfide Cross-Linked pHDP-PEG-FA[−] Decationized Polyplexes, Through a 3-Step Process: 1. Charge-Driven Condensation; 2. Stabilization through Disulfide Crosslinking; 3. Polyplex Decationization (Adapted from Novo et al.¹⁹)


interchain disulfide cross-linking reaction in the core of the polyplexes using the dithiol DDTT.

The block copolymer compositions were determined by ^1H NMR and UV spectroscopy¹⁹ (Table 1). As previously shown, the copolymer composition as determined by NMR analysis was close to the feed ratio of HPMA-DMAE/PDTEMA. Both polymers pHDP-PEG-FA and pHDP-PEG had similar M_n as determined by NMR analysis. UV spectroscopy showed that the molarity of PDS groups per weight of polymer was similar for both polymers. GPC results indicated that polymer characteristics, such as M_w , PDI, and dn/dc , are similar for both polymers. Using the polymer characterization data, it can be calculated that the synthesized polymers contained approximately one disulfide cross-linking point (PDTEMA) per 6.5 cationic units (HPMA-DMAE).

Preparation and Stability of pHDP-PEG(FA) Polyplexes. Polyplexes of pHDP-PEG and pHDP-PEG-FA were formed through the 3-step process as schematically shown in Scheme 2 and as also reported in our previous work.¹⁹ The preparation of FA-targeted decationized polyplexes started with the synthesis of the cationic block copolymer p(HPMA-DMAE-co-PDTEMA-b-PEG-FA) (pHDP-PEG-FA[+]), which contains FA at the PEG distal end. The block copolymer was subsequently used to complex pDNA via electrostatic interactions and interchain disulfide cross-linking of the polyplexes was performed via thiol–disulfide exchange reaction between a short dithiol (DDTT) and the PDS groups of the PDTEMA units present in the polymer backbone. In order to achieve polyplex core-cross-linking DDTT was added at a molar equivalent of thiol groups to the PDS groups of the polymer. Structural stabilization and pDNA entrapment occurred due to the interchain disulfide cross-links in the

Scheme 3. Synthesis of pHDP-PEG-FA and pHDP-PEG


pHPMA-DMAE core. Decationization was performed by removal of the DMAE cationic side groups linked to the HPMA backbone via carbonate ester bond at pH 8.5, to form p(HPMA-co-PDTEMA)-b-PEG-FA (pHP-PEG-FA[-]) decationized polyplexes, containing the folate moiety at their PEG shell.

The biophysical properties of both pHDP-PEG and pHDP-PEG-FA polyplexes obtained by DLS and zeta potential measurements are given in Table 2. Polyplexes were first

Table 2. Particle z-Average Diameter (Z-avg) and Polydispersity Index (PDI) Determined by DLS and Particle Charge (ζ Pot) Determined by Zeta Potential Measurements of pHDP-PEG-FA and pHDP-PEG Based Polyplexes, at Different Stages of Preparation^a

polyplexes	pHDP-PEG-FA		
	DLS		Zetasizer
	Z-ave (nm)	PDI	ζ Pot (mV)
After cross-linking	142 \pm 8	0.130 \pm 0.046	6.5 \pm 1.4
After decationization	152 \pm 8	0.099 \pm 0.011	-12.4 \pm 0.6
polyplexes	pHDP-PEG		
	DLS		Zetasizer
	Z-ave (nm)	PDI	ζ Pot (mV)
After cross-linking	127 \pm 5	0.162 \pm 0.028	10.2 \pm 3.1
After decationization	132 \pm 8	0.190 \pm 0.064	-5.3 \pm 1.5

^aPolyplexes were prepared at N/P = 4 and a pDNA concentration of 50 μ g/mL. Results are expressed as mean \pm SD (n = 3).

prepared by complexing the polymer with pDNA at N/P = 4, which was selected because it was shown in our previous study that the pHDP-PEG particles prepared at this N/P had best biophysical and *in vitro* transfection properties.¹⁹ Both polymers formed nanosized polyplexes with a diameter of 127 \pm 5 nm for pHDP-PEG[+] polyplexes and 142 \pm 8 nm for pHDP-PEG-FA[+] polyplexes. Also, both polyplexes showed a positive zeta potential; however, the zeta potential of the pHDP-PEG-FA[+] polyplexes (+6.5 \pm 1.4 mV) was slightly lower than that observed for the pHDP-PEG[+] (+10.2 \pm 3.1). This difference in zeta potential has been previously observed for folate conjugated polyplexes^{27,40} and is ascribed to of the presence of carboxylic acid groups of folate at the shell of the polyplexes, which are negatively charged at pH 7 and therefore lower the zeta potential of the particles. After cross-linking, removal of DMAE cationic groups from the polyplexes was performed by incubation of the polyplex dispersions at pH 8.5 and 37 $^{\circ}$ C for 6 h to yield decationized polyplexes pHP-PEG[-] with and without FA groups. DLS results showed that after decationization the polyplex diameter slightly increased from 142 \pm 8 to 152 \pm 8 nm for pHP-PEG-FA[-] polyplexes and from 127 \pm 5 to 132 \pm 8 nm for pHP-PEG[-]. Most likely, loss of electrostatic interactions between the polymer and pDNA resulted in hydration and slight swelling of the polyplex core.

Importantly, the zeta potentials of the polyplexes dropped from slightly positive to negative values after decationization (-12.4 \pm 0.6 mV for pHP-PEG-FA[-] and -5.3 \pm 1.5 mV for pHP-PEG[-] and polyplexes). The change in the zeta potential upon decationization procedure is due to the loss of the cationic DMAE groups from the pHDP core. Again, a lower zeta potential was observed for the folate targeted polyplexes; therefore, after decationization the folic acid groups are most likely present on the surface of the polyplexes.

When pHP-PEG-FA[-] decationized polyplexes were incubated in the presence of a reductive agent (10 mM DTT) (Figure 4) and subjected to a gel retardation assay, release of

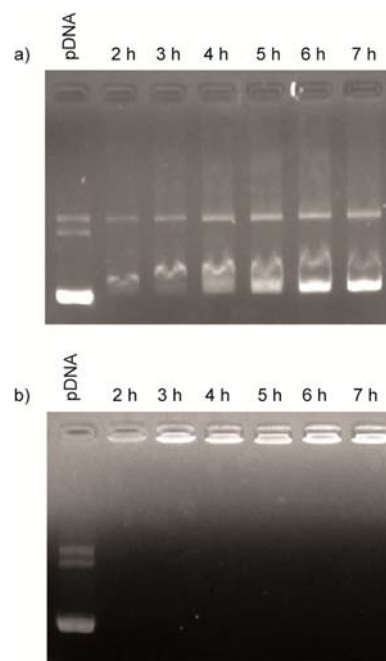


Figure 4. Agarose gel retardation assay of pHP-PEG-FA[-] (a) and pHDP-PEG-FA[+] (b) polyplexes upon incubation with 10 mM DTT in HBS at 37 $^{\circ}$ C, for 2 h; 3 h; 4 h; 5 h; 6 h; 7 h.

pDNA was observed (Figure 4a), whereas for their cationic counterparts, pDNA remained stably encapsulated in the polyplexes (Figure 4b). This confirms the redox triggered release previously observed for pHP-PEG[-] polyplexes.¹⁹

In Vitro Evaluation of the pHP-PEG-FA[-] Polyplexes in Cell Cultures. The cellular uptake of pHP-PEG[-] (decationized), pHP-PEG-FA[-] (targeted and decationized), and pHDP-PEG[+] (cationic) polyplexes was evaluated, in the presence of serum, in two folate overexpressing cell lines, HeLa (Figure 5a) and OVCAR-3 (Figure 5b), and in a folate nonexpressing cell line A549 (Figure 5c).⁴¹ The polyplexes were prepared at low concentration with Cy5 labeled pDNA and the degree of uptake was accessed by flow cytometry 2 h after incubation with the cells at 37 $^{\circ}$ C. In the case of the folate overexpressing cell lines, the targeted decationized polyplexes pHP-PEG-FA[-] were taken up by cells to a much higher extent (a factor 3–4) than the nontargeted decationized pHP-PEG[-] polyplexes. In the case of A549 cell line, the pHP-PEG-FA[-] polyplexes had an almost insignificant degree of uptake, even lower than pHP-PEG[-] polyplexes. The observed relatively lower uptake of the targeted formulation is probably due to a more negative zeta potential of pHP-PEG-FA[-] polyplexes, that results in low nonspecific interaction with cells. These results clearly show that the high uptake of pHP-PEG-FA[-] polyplexes by the folate receptor overexpressing cell lines, HeLa and OVCAR-3, is due to the presence of folate moieties on the surface of the polyplexes and not due to small differences in their biophysical properties. It should be stressed again that nontargeted cationic polyplexes pHDP-PEG[+] showed a very high degree of uptake in the cell lines tested, once more confirming that polyplexes based on polycations, even when PEGylated, are not suitable for a targeted therapy because

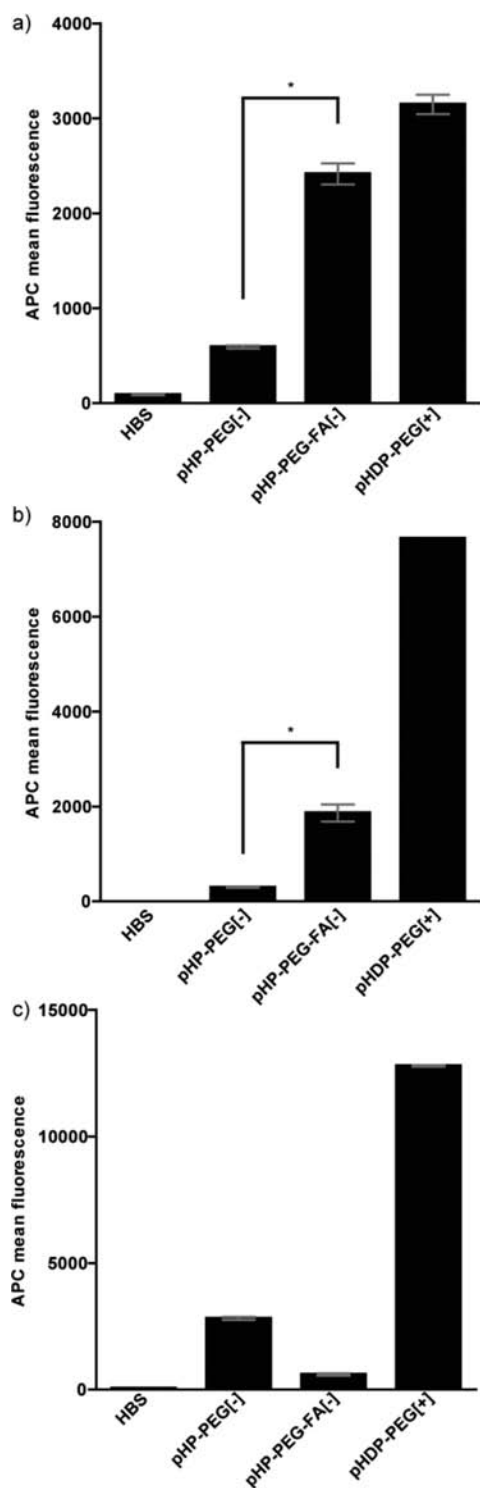


Figure 5. Mean fluorescence intensity per cell after different Cy5-pDNA polyplexes were incubated with HeLa cells (FR +) (a), OVCAR-3 cells (FR +) (b), and A549 (FR -) (c) for 2 h at 37 °C (pDNA dose was 10 $\mu\text{g}/\text{mL}$, 10%wt Cy5 pDNA). Results are expressed as mean \pm SD ($n = 3$). * $p < 0.01$ (t test).

specific uptake via the receptor that matches the used targeting ligand is most likely overruled by the high degree of nonspecific uptake.

Transfection efficiency of the decationized polyplexes with and without FA was evaluated using polyplexes of N/P = 4 and at pDNA doses of 3 and 5 μg per well. The transfection

efficiency of the FA-decorated and control polyplexes in OVCAR-3 (FR +) cells was studied in the absence of folic acid (medium and serum).⁴² As control, transfection was also evaluated in folate saturated medium (1 mM).

Folate-targeted nanoparticles or conjugates are known to interact with high affinity to the folate receptor of the cells and be internalized by receptor-mediated endocytosis.⁴³ The polyplexes which end up in endosomes after cellular uptake need to escape from the endosomes before lysosomal digestion occurs.^{3,44–46} PHP-PEG[-] based polyplexes do not possess endosomal escape functionalities,¹⁹ and therefore chloroquine, a known agent to induce endosomal escape,⁴⁷ was added to the transfection medium. As positive control, ExGen 500 (1-PEI) based polyplexes were used.³⁸ The polyplexes were first incubated with the cells for 4 h at 5 °C, allowing binding of polyplexes to the cells and minimizing artifact effects on transfection such nonspecific cell binding due to particle sedimentation and/or excessive contact time of the polyplexes with the cells. Subsequently, medium containing polyplexes which have not interacted with the cells was washed away, and the cells were incubated at 37 °C to allow active internalization of the polyplexes firmly interacting with the cells. Polyplexes were added to the cells in a relatively high dose to maximize the number of specific polyplex interactions per cell. Figure 6

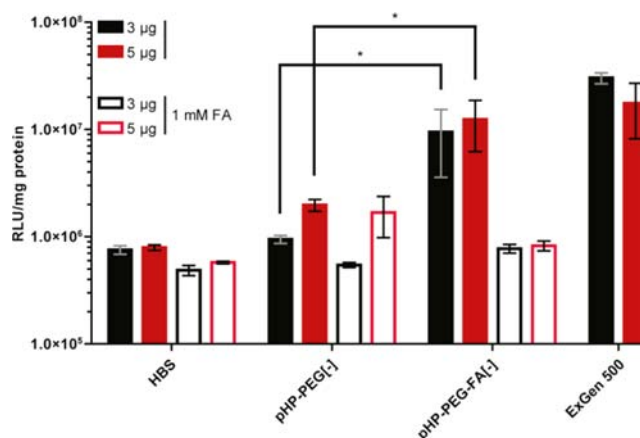


Figure 6. Effect of pDNA dose (3 and 5 μg of pDNA/well; final FA concentration is 2.26 μM and 3.37 μM , respectively) and on luciferase expression levels of OVCAR-3 cells (FR +) incubated with different polyplexes (N/P = 4, pHP-PEG[-] and pHP-PEG-FA[-]). OVCAR-3 cells were incubated with polyplex dispersions at 5 °C, followed by removal of unbound polyplexes, and incubated at 37 °C for 4 h for transfection. Polyplexes were prepared with an N/P = 4. A RLU is unit equivalent to 0.52 pg of luciferase ($r^2 = 0.99$, $n = 3$). Results are expressed as mean \pm SD ($n = 4$). * $p < 0.05$ (t test).

shows that targeted pHP-PEG-FA[-] polyplexes exhibited a transfection level in OVCAR-3 cells close to ExGen. More importantly, an increasing dose was not detrimental for transfection, emphasizing the safety of a neutral polymer based system. It should be noted that transfection levels of pHP-PEG-FA[-] are relatively low when compared to previously shown transfection levels of ExGen in its optimal transfection conditions.¹ However, in the same setup conditions, where nonspecific internalization was minimized, PEI polyplexes had comparable transfection efficiency to pHP-PEG-FA[-] polyplexes. Often transfection levels of PEI polyplexes are unrealistically high since they arise from their significant aggregation in biological media. Aggregation of PEI

formulations (>500 nm) is known to be one of the key factors for its high transfection efficiency *in vitro*.³⁸

Figure 6 also shows that, when the same cells were transfected with folate-decorated polyplexes in folate saturated medium, the transfection was completely blocked. On the other hand, the transfection levels of cells incubated with nontargeted polyplexes did not decrease in folate saturated medium, which was particularly evident for the highest pDNA dose (5 μ g). These results reveal that the decrease in transfection for pHP-PEG-FA[-] polyplexes in folate saturated medium is due to saturation of the folate receptors with free folate, making them inaccessible for interaction with the folate-decorated polyplexes. In folate saturated medium, some degree of transfection was observed for pHP-PEG[-] polyplexes, in contrast to pHP-PEG-FA[-] particles, in which no detectable luciferase expression was observed at both pDNA doses. This result is in accordance with the uptake observed for folate nonexpressing cell line A549 (Figure 2c), where nonspecific uptake is higher for nontargeted polyplexes. The low degree of transfection observed for pHP-PEG[-] polyplexes is also in accordance with the findings previously observed in HeLa and A549,¹⁹ where the lack of cellular uptake greatly contributed the low transfection activity of these polyplexes.

In the absence of folic acid in the medium, the difference in transfection activity between targeted pHP-PEG-FA[-] and nontargeted pHP-PEG[-] polyplexes is close to 1 order of magnitude higher for the targeted systems. Several reports have shown the improvement of transfection efficiency by folate targeting of polyplexes,^{48–50} and our results are in line with these findings. In the case of our system, folate targeting triggers transfection with very high specificity for target cells. Furthermore, Salvati et al.⁵¹ reported that targeting specificity can be lost by unspecific interactions with components from biological fluids with nanoparticles, the lack of cationic charges in the decationized polyplexes is expected to further reduce the occurrence of unspecific interactions, improving in this way the targeting specificity.

The cytotoxicity of targeted pHP-PEG[-] polyplexes was evaluated using the XTT assay and compared with that of nontargeted pHP-PEG[-] and cationic nontargeted pHDP-PEG[+] polyplexes. Polyplexes were prepared at an N/P = 4 and tested at different pDNA doses (0.25, 1, and 3 μ g pDNA per well, Figure 7) and incubated HeLa cells for 24 h. This figure shows that high metabolic activity was found for the cells incubated with both targeted and nontargeted polyplexes. The outcome of the study reveals that, even after extensive uptake of the decationized polyplexes, metabolic activity of the cells was not affected, confirming the safety profile that was previously found for nontargeted decationized systems in HeLa cells.¹⁹

CONCLUSION

Folate targeted polyplexes based on the hydrophilic and noncharged pHP-PEG polymer were prepared using the cationic pHDP-PEG-FA polymer as precursor. The introduction of the folate targeting units at the surface of decationized polyplexes did not alter significantly their properties like triggered release under intracellular-mimicking reducing conditions, polyplex biophysical properties, or the safety profile of this system. Importantly, uptake of FA targeted decationized polyplexes by folate receptor overexpressing cell lines, HeLa and OVCAR-3, was significantly higher when compared to its nontargeted counterpart. By contrast, uptake studies with the folate receptor negative cell line, A549, showed a slightly lower

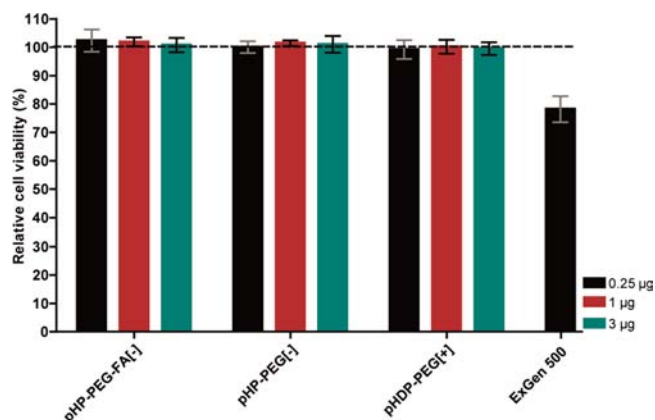


Figure 7. Effect of pDNA dose (0.25, 1, and 3 μ g of pDNA/well) on cell viability of HeLa cells (FR+) for the different polyplexes (pHP-PEG[-], pHP-PEG-FA[-], and pHDP-PEG[+]). HeLa cells were transfected in the presence of 10% serum. Polyplexes were prepared with a starting N/P = 4 and incubated with the cells for 24 h at 37 $^{\circ}$ C. Results are expressed as mean \pm SD (n = 3).

uptake for targeted polyplexes. In line with the uptake studies, transfection efficiency determined using OVCAR-3 showed striking differences between pHP-PEG-FA[-] and pHP-PEG[-] polyplexes. When the medium was saturated with free folic acid the transfection activity of targeted polyplexes was reduced to the level of the nontargeted system.

The results presented in this paper demonstrate that the introduction of folic acid moiety at the surface of decationized polyplexes allow the preparation of a targetable polyplex formulation that is highly promising for site-specific gene therapy after intravenous administration.

MATERIALS AND METHODS

Materials. *N*-(2-Hydroxypropyl)methacrylamide (HPMAm) was obtained from Zentiva a.s. (Prague, Czech Republic). Carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester (HPMA-DMAE) and *N*-[2-(2-pyridyldithio)]ethyl methacrylamide (PDTEMA) were synthesized as previously described.¹⁹ NH_2 -PEG-OH (M_w 5000 Da) was obtained from NOF corporation (Kyoto, Japan). pCMV_Luc plasmid, encoding for firefly luciferase, with human cytomegalovirus promoter (CMV), was amplified with competent *E. coli* DH5 α and purified with NucleoBond (Macherey-Nagel, Bioke, Leiden, The Netherlands). pCMV Luc was purchased from the Plasmid Factory (Bielefeld, Germany). Agarose MP was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Exgen 500 (22 kDa, I-PEI) and 6 \times DNA Loading Dye were purchased from Fermentas (St. Leon-Roth, Germany). HeLa (human epithelial ovarian carcinoma cell line), OVCAR-3 (ovarian carcinoma cell line), and A549 (human lung epithelial carcinoma cell line) cells were obtained from the American Type Culture Collection (ATCC) (Maryland, USA). Dulbecco's Modified Eagle Medium with 3.7 g/L sodium bicarbonate, 1 g/L L-glucose, L-glutamine (DMEM), RPMI 1640, Ham's F-12 Nutrient Mixture (F-12), phosphate buffered saline (PBS), fetal bovine serum (FBS), antibiotics/antimycotics (penicillin, streptomycin sulfate, amphotericin B), and trypsin/EDTA 10 \times were purchased from PAA Laboratories GmbH (Pasching, Austria). Folate free RPMI-1640 medium and dialyzed FBS was purchased from Gibco (Breda, The Netherlands).

Luciferase assay kit and QuantiLum recombinant luciferase were obtained from Promega (Leiden, The Netherlands). 2,4,6-Trinitrobenzene sulfonic acid (TNBSA) and Micro BCA Protein Assay Kits were purchased from Pierce (Etten-Leur, The Netherlands). LabelIT Cy5 Nucleic Acid Labeling Kit was purchased from Mirus Bio (Madison, WI, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Synthesis of FA-NHS. FA-NHS was prepared according to previously described procedures, with small changes.^{27,28} Folic acid (FA) (1 g, 2.27 mmol) was dissolved in 15 mL DMSO at 40 °C. Next, the solution was cooled to room temperature and 4-dimethylaminopyridine (DMAP) (0.056 g, 0.45 mmol) and *N*-hydroxysuccinimide (NHS) (0.523 g, 4.54 mmol) were added. After dissolution of both compounds, *N,N'*-dicyclohexylcarbodiimide (DCC) (0.468 g, 2.27 mmol) was slowly added to the reaction mixture under vigorous stirring. The reaction mixture was kept in the dark for 24 h under an N₂ atmosphere. The solution was consecutively filtered to remove precipitated dicyclohexylurea (DCU), followed by precipitation of FA-NHS in a mixture of ether/acetone 85/15 (500 mL). The precipitated product was washed and collected by filtration. The product was further dried in a vacuum oven for 2 days.

Synthesis of (FA-PEG)₂-ABCPA (Scheme 1). *Boc Protection of NH₂-PEG-OH.* NH₂-PEG-OH (5000 Da) (1 g, 0.20 mmol) was dissolved in 10 mL DCM. Next, di-*tert*-butyl dicarbonate (t-Boc) (87.3 mg, 0.40 mmol) was added to the solution. The reaction mixture was stirred for 24 h under an N₂ atmosphere. Next, DCM was partially evaporated under reduced pressure and the obtained product (Boc-NH-PEG-OH) was precipitated in 50 mL diethyl ether. The product was dissolved in DCM and precipitated diethyl ether (3 times).

Synthesis of (Boc-NH-PEG)₂-ABCPA. The synthesis and characterization of (Boc-NH-PEG)₂-ABCPA was based on the (mPEG)₂-ABCPA macroinitiator synthesis as previously described,^{29,30} with some modifications.

Boc-NH-PEG-OH (1020.2 mg, 0.2 mmol) was introduced into a round bottomed-flask and dried overnight in a vacuum oven. After dissolution of PEG in 7 mL of DCM, 1 mL of 0.1 M 4,4'-azobis(4-cyanovaleic acid) (ABCPA) solution (0.10 mmol) in DCM/THF (1/1 v/v) was added to the PEG solution. Next, 1 mL of 0.03 M 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) solution in DCM (0.03 mmol) was added to the mixture and 1 mL THF was added to adjust the PEG concentration to ~100 mg/mL. The mixture was cooled on ice, and 1 mL 0.3 M DCC solution (0.3 mmol) in DCM/THF (1/1 v/v) was slowly added. The reaction was carried at room temperature for 48 h under an N₂ atmosphere.

The formed DCU was removed by centrifugation, followed by filtration through a 0.2 μm nylon filter. The product (Boc-NH-PEG)₂-ABCPA was further purified by precipitation in diethyl ether, followed by dissolution in DCM and precipitation in diethyl ether (2 times).

Deprotection of (Boc-NH-PEG)₂-ABCPA. (Boc-NH-PEG)₂-ABCPA (200 mg, 19.1 μmol) was deprotected in 2 mL DCM/TFA 1/1 (v/v) mixture for 4 h at room temperature. Next, the solvent was partially evaporated under reduced pressure and the product (TFA-NH₂-PEG)₂-ABCPA was precipitated in diethyl ether, followed by dissolution in DCM and precipitation in diethyl ether (3 times).

Synthesis of (FA-PEG)₂-ABCPA. FA-NHS (48.46 mg, 90 μmol) was dissolved in 1.5 mL DMSO and 21 μL of triethylamine (150 μmol) was subsequently added. Next, a

solution of (TFA-NH₂-PEG)₂-ABCPA (154.2 mg, 15 μmol) in 1.5 mL DMSO was slowly added to this reaction mixture. The reaction was allowed to proceed for 36 h under an N₂ atmosphere in the dark.

The product was purified by dialysis against DMSO for 24 h (MWCO 6000–8000 Da), followed by a gradual exchange of the dialysis medium to 2 mM NaOAc pH 5. The purified product was filtered over a 0.2 μm nylon filter and freeze-dried.

Synthesis of pHDP-PEG-FA and pHDP-PEG. Free radical polymerization using (FA-PEG)₂-ABCPA or (Boc-NH-PEG)₂-ABCPA as macroinitiator was performed to synthesize p(HPMA-DMAE-co-PDTEMA)-b-PEG-FA (pHDP-PEG-FA) or p(HPMA-DMAE-co-PDTEMA)-b-PEG-Boc (pHDP-PEG), respectively. The polymers were synthesized using a HPMA-DMAE-to-initiator ratio (M/I) of 200 (mol/mol). The feed ratio HPMA-DMAE/PDTEMA was 1/0.2 (mol/mol). The polymerization was carried at 70 °C for 24 h in DMSO under an N₂ atmosphere, using 2.5 μmol macroinitiator and monomer concentration of 1 M. After polymerization, the polymers were precipitated in diethyl ether, dialyzed against 5 mM NH₄OAc buffer pH 5.0 for 3 days at 4 °C (MWCO 6000–8000), and collected by freeze-drying.

Polymer Characterization. *Gel Permeation Chromatography (GPC) Characterization of the Polymers.* Analysis of the (FA-PEG)₂-ABCPA macroinitiator was performed using a Waters System (Waters Associates Inc., Milford, MA) with refractive index (RI) and UV detector using two serial Plgel 5 μm MIXED-D columns (Polymer Laboratories) and DMF containing 10 mM LiCl as eluent. The flow rate was 0.7 mL/min (50 min run time) and the temperature was 40 °C.³¹ The molecular weights of the synthesized polymers were determined by GPC analysis using a Viscotek-GPCmax (Viscotek, Oss, The Netherlands) light scattering/viscosimetric detection system, first by determining the dn/dc of the different polymers and subsequently by using two Ultrahydrogel 1000 7.8 × 300 mm in series with Ultrahydrogel 6 × 40 guard column and 0.3 M NaOAc pH 4.4 as eluent.³² The flow rate was 0.7 mL/min (run time was 50 min) and the temperature was 30 °C. Data from the laser photometer (λ = 670 nm) (right (90°) and low (7°) angle light scattering), RI, and viscosity detector were integrated using OmniSEC software to calculate the number and weight average molecular weight (*M_n*, *M_w*) and polydispersity index (PDI; (*M_w*/*M_n*)). UV detection at 280 nm, specific for the pyridyldisulfide (PDS) group, was also applied.³³ PEO (*M_n* = 18.1 kDa, *M_w* = 18.9 kDa, Malvern (Worcestershire, UK)) was used for calibration.

¹H NMR Characterization of the Polymers. The copolymer composition of the different polymers dissolved in DMSO-*d*₆ was determined by ¹H NMR analysis performed using a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA). The copolymer composition and its *M_n* were determined as previously described.¹⁹

Preparation of Decationized Polyplexes. The preparation of decationized polyplexes was essentially performed as previously described (Scheme 2).³⁴ Briefly, to prepare a dispersion of 50 μg/mL pDNA polyplexes, 100 μL of polymer solutions in 20 mM Tris-HCl pH 8.5 buffer (pHDP-PEG (nontargeted) or pHDP-PEG-FA (folate targeted)) at N/P = 4 (N, molarity positively charged amines from polymer; P, molarity of negatively charged phosphates from pDNA) were mixed with 200 μL pDNA (75 μg/mL). After 10 min at room temperature, polyplexes were cross-linked by addition of 3,6-dioxa-1,8-octane-dithiol (DODT) corresponding with a molar

equivalent of DODT thiol groups to PDS groups of the polymer.

Polyplexes were decationized by incubation of the polyplex dispersions at 37 °C and pH 8.5 for 6 h, to hydrolyze the cationic dimethylaminoethanol (DMAE) groups from the polymers.¹⁹ Next, the ionic strength and pH were adjusted to physiological conditions (150 mM, pH 7.4).

Given the fact that the side products from cross-linking and decationization, (2-mercaptopyridine and DMAE) possess high cellular tolerance, as previously demonstrated,^{19,35,36} polyplexes were directly used without purification procedures.

Gel Retardation Assay. Decationized pHP-PEG-FA[-] and cationic pHDP-PEG-FA[+] polyplexes were prepared at N/P = 4 and subsequently incubated with 10 mM dithiothreitol (DTT) (as reducing agent) at 37 °C. At specific incubation time points, samples of 20 μ L polyplex dispersion in HEPES buffered saline (HBS) (20 μ g/mL of pDNA) were collected. Samples were then mixed with 4 μ L 6 \times DNA Loading Dye and loaded into 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer containing 0.5 μ g/mL ethidium bromide and run at 120 V for 40 min pDNA was detected using a Gel Doc XR+ system (BioRad Laboratories Inc., Hercules, CA) with Image Lab software.

Cell Culture. HeLa cells (FR +; folate receptor positive cells) were cultured in DMEM supplemented with antibiotics/antimycotics and 10% FBS. OVCAR-3 cells (FR +) were cultured in RPMI 1640 supplemented with antibiotics/antimycotics, bovine insulin (0.01 mg/mL), sodium pyruvate (1 mM), and 20% FBS. A549 cells (FR -; folate receptor negative cells) were cultured in Ham's F-12 supplemented with antibiotics/antimycotics and 10% FBS. Cells were maintained at 37 °C in a 5% CO₂ humidified air atmosphere.

All *in vitro* experiments were performed in the presence of 20% serum at every stage of the *in vitro* experimental setup.

Cellular Uptake of Polyplexes. For uptake studies, 30,000 HeLa, 90,000 OVCAR-3, or 36,000 A549 cells/well were seeded into 24-well culture plates. pDNA was labeled with Cy5 according to the manufacturer's protocol using a Label IT Nucleic Acid Labeling Kit and polyplexes were prepared at a final pDNA concentration of 10 μ g/mL, containing 10 wt % labeled pDNA. Polyplexes were incubated with HeLa, OVCAR-3, or A549 cells for 2 h at 37 °C in the presence of serum (10% FBS for HeLa and A549 cells, 20% FBS for OVCAR-3 cells) and washed 3 times (PBS, glycine buffer (0.2 M glycine, 0.15 M NaCl, pH 3), and PBS). Glycine buffer was used to remove adsorbed particles on the surface of the cells.³⁷ The degree of cellular uptake (Cy5-positive cells) was determined by flow cytometry.

Flow Cytometry. The cells adhering to the well plate were washed, trypsinized (50 μ L 1 \times trypsin/EDTA), and resuspended in FBS supplemented medium (150 μ L). The cells were subsequently transferred into a round-bottom 96-well plate and centrifuged for 5 min at 250 \times g and 4 °C. After medium removal, the cells were resuspended in 100 μ L phosphate-buffered albumin (PBA; 1% w/v albumin in PBS) and fixed with 100 μ L of 10% formalin. Samples were analyzed by flow cytometry using a FACSCantoII (Becton and Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW He-Ne laser. A number of 10,000 cell events were recorded per sample to determine the degree of uptake for Cy-5 positive cells (APC channel).

Statistical analyses were performed with the software GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California, USA).

Polyplex transfection activity. The transfection activity of the different polyplex formulations was determined by luciferase assay essentially as described by van Gaal et al.³⁸ OVCAR-3 cells were plated into 96-well plates 48 h prior to transfection (15,000 cells per well). Before addition of the polyplex dispersions to the cells, the culture medium was replaced by folate-free RPMI 1640 supplemented with 25% dialyzed FBS, 250 μ M chloroquine with or without 1.25 mM folic acid. Next, 25 μ L of polyplex dispersions containing 120 μ g/mL or 200 μ g/mL of pDNA were added per well and the plates were immediately placed on ice for 4 h. Cells were washed 3 \times with PBS, and folate-free RPMI 1640 supplemented with 20% FBS, 200 μ M chloroquine and with or without 1 mM folic acid was added to the cells. Cells were incubated for 24 h at 37 °C and the culture medium was replaced with fresh RPMI containing 20% FBS. Cells were additionally incubated for 24 h. Polyplexes based on Exgen 500 at N/P = 6 were used as positive control.³⁸

Luciferase expression assay was performed 48 h after transfection. Cells were washed with 100 μ L of cold PBS and lysed with 50 μ L of lysis buffer (Reporter Lysis Buffer 5 \times (Promega), diluted in mQ H₂O). A freeze-thaw cycle was performed by incubating the cells for 1 h at -80 °C. Next, 10 μ L of cell lysate was mixed with 50 μ L of Luciferase Assay Reagent (Promega); after 2 s luminescence was measured for 10 s using a FLUOstar OPTIMA microplate, equipped with a luminescence light guide (BMG LabTech, Germany). The obtained luciferase activity was normalized to the amount of protein in the lysates, determined by Micro BCA Protein Assay. Results are expressed as relative light units (RLU) per mg of cellular protein. RLU were quantified using QuantiLum recombinant luciferase standards (1 \times 10² to 1 \times 10⁶ pg) in 10 μ L 1 \times Reporter Lysis Buffer containing 1 mg/mL bovine serum albumin (BSA), following the suppliers recommendation. Linear regression was performed with GraphPad Prism 5.

Statistical analyses were performed with the software GraphPad Prism 5.

XTT Assay. Cell viability was analyzed using the XTT assay which determines the metabolic activity of HeLa cells upon incubation with polyplex dispersions. HeLa cells were seeded 24 h prior to transfection into 96-well plates (5000 HeLa cells/well). Immediately prior to transfection, the culture medium was replaced with DMEM supplemented with 12.5% FBS. Next, samples of 25 μ L polyplex dispersions containing 10, 40, or 150 μ g/mL of pDNA were added per well. Medium was replaced after 24 h and the XTT assay was performed 48 h after transfection. Two hours before performing the XTT assay, the medium was refreshed. After adding 50 μ L of a freshly prepared XTT solution (25 μ M N-methyl dibenzopyrazine methylsulfate (PMS) and 1 mg/mL 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in plain RPMI 1640 medium) per well, the cells were incubated for 1 h at 37 °C in a CO₂-incubator. Next, absorbance was measured at 490 nm with a reference wavelength of 655 nm. Cell viability is expressed as relative metabolic activity normalized against cells cultured with HBS without polyplex dispersions.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +31 30 253 6964; fax: +31 30 251 7839. E-mail address: w.e.hennink@uu.nl.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABCPA, 4,4'-azobis(4-cyanovaleric acid); CMV, human cytomegalovirus promoter; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, dicyclohexylurea; DMAE, dimethylaminoethanol; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DODT, 3,6-dioxo-1,8-octane-dithiol; DPTS, 4-(dimethylamino)pyridinium 4-toluenesulfonate; EDTA, ethylenediaminetetraacetic acid; EPR, enhanced permeation and retention; FA, folic acid; FBS, fetal bovine serum; FR, folate receptor; GPC, gel permeation chromatography; HBS, HEPES buffered saline; HPMA-DMAE, 1-methyl-2-(2-methacryloylamino)-ethyl ester; HPMAm, *N*-(2-hydroxypropyl)-methacrylamide; M_n , number average molecular weight; M_w , weight average molecular weight; MWCO, molecular weight cutoff; NaOAc, sodium acetate; NH_4OAc , ammonium acetate; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PDI, polydispersity index; pDNA, plasmid DNA; PDS, pyridyldisulfide; PDTEMA, *N*-[2-(2-pyridyldithio)]ethyl methacrylamide; PEG, poly(ethylene glycol); PEI, polyethylenimine; PEO, poly(ethylene oxide); PMS, *N*-methyl dibenzopyrazine methylsulfate; RI, refractive index; RLU, relative light units; t-Boc, tert-butyl dicarbonate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TNBSA, (2,4,6-trinitrobenzene sulfonic acid); XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide

REFERENCES

- (1) Seymour, L. W., and Thrasher, A. J. (2012) Gene therapy matures in the clinic. *Nat. Biotechnol.* 30, 588–593.
- (2) Thomas, C. E., Ehrhardt, A., and Kay, M. A. (2003) Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* 4, 346–358.
- (3) Mintzer, M. A., and Simanek, E. E. (2008) Nonviral vectors for gene delivery. *Chem. Rev.* 109, 259–302.
- (4) Li, W., and Szoka, F. C., Jr. (2007) Lipid-based nanoparticles for nucleic acid delivery. *Pharm. Res.* 24, 438–449.
- (5) Luten, J., van Nostrum, C. F., De Smedt, S. C., and Hennink, W. E. (2008) Biodegradable polymers as non-viral carriers for plasmid DNA delivery. *J. Controlled Release* 126, 97–110.
- (6) Wong, S. Y., Pelet, J. M., and Putnam, D. (2007) Polymer systems for gene delivery—Past, present, and future. *Prog. Polym. Sci.* 32, 799–837.
- (7) Mastrobattista, E., van der Aa, M. A. E. M., Hennink, W. E., and Crommelin, D. J. A. (2006) Artificial viruses: a nanotechnological approach to gene delivery. *Nat. Rev. Drug Discovery* 5, 115–121.
- (8) Yousefi, A., Storm, G., Schiffelers, R., and Mastrobattista, E. (2013) Trends in polymeric delivery of nucleic acids to tumors. *J. Controlled Release* 170, 209–218.
- (9) Midoux, P., Pichon, C., Yaouanc, J.-J., and Jaffrès, P.-A. (2009) Chemical vectors for gene delivery: a current review on polymers,

peptides and lipids containing histidine or imidazole as nucleic acids carriers. *Br. J. Pharmacol.* 157, 166–178.

(10) Chollet, P., Favrot, M. C., Hurbin, A., and Coll, J.-L. (2002) Side-effects of a systemic injection of linear polyethylenimine–DNA complexes. *J. Gene Med.* 4, 84–91.

(11) Ward, C. M., Read, M. L., and Seymour, L. W. (2001) Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood* 97, 2221–2229.

(12) Moghimi, S. M., Symonds, P., Murray, J. C., Hunter, A. C., Debska, G., and Szewczyk, A. (2005) A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol. Ther.* 11, 990–995.

(13) Maeda, H. (2010) Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjugate Chem* 21, 797–802.

(14) Fang, J., Nakamura, H., and Maeda, H. (2011) The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv. Drug Delivery Rev.* 63, 136–151.

(15) Bae, Y. H., and Park, K. (2011) Targeted drug delivery to tumors: Myths, reality and possibility. *J. Controlled Release* 153, 198–205.

(16) Verbaan, F. J., Oussoren, C., Snel, C. J., Crommelin, D. J. A., Hennink, W. E., and Storm, G. (2004) Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. *J. Gene Med.* 6, 64–75.

(17) Ogris, M., Brunner, S., Schuller, S., Kircheis, R., and Wagner, E. (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* 6, 595–605.

(18) Harada-Shiba, M., Yamauchi, K., Harada, A., Takamisawa, I., Shimokado, K., and Kataoka, K. (2002) Polyion complex micelles as vectors in gene therapy – pharmacokinetics and in vivo gene transfer. *Gene Ther.* 9, 407–14.

(19) Novo, L., van Gaal, E. V. B., Mastrobattista, E., van Nostrum, C. F., and Hennink, W. E. (2013) Decationized crosslinked polyplexes for redox-triggered gene delivery. *J. Controlled Release* 169, 246–256.

(20) Lee, M. H., Yang, Z., Lim, C. W., Lee, Y. H., Dongbang, S., Kang, C., and Kim, J. S. (2013) Disulfide-cleavage-triggered chemosensors and their biological applications. *Chem. Rev.* 113, 5071–5109.

(21) Meng, F., Hennink, W. E., and Zhong, Z. (2009) Reduction-sensitive polymers and bioconjugates for biomedical applications. *Biomaterials* 30, 2180–2198.

(22) Hilgenbrink, A. R., and Low, P. S. (2005) Folate receptor-mediated drug targeting: From therapeutics to diagnostics. *J. Pharm. Sci.* 94, 2135–2146.

(23) Teng, L., Xie, J., Teng, L., and Lee, R. J. (2012) Clinical translation of folate receptor-targeted therapeutics. *Expert Opin. Drug Delivery* 9, 901–8.

(24) Lu, Y., and Low, P. S. (2002) Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Delivery Rev.* 54, 675–693.

(25) Lu, Y., and Low, P. S. (2012) Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Delivery Rev.* 64 (Supplement), 342–352.

(26) Dohmen, C., Edinger, D., Fröhlich, T., Schreiner, L., Lächelt, U., Troiber, C., Rädler, J., Hadwiger, P., Vornlocher, H.-P., and Wagner, E. (2012) Nanosized multifunctional polyplexes for receptor-mediated siRNA delivery. *ACS Nano* 6, 5198–5208.

(27) van Steenis, J. H., van Maarseveen, E. M., Verbaan, F. J., Verrijck, R., Crommelin, D. J. A., Storm, G., and Hennink, W. E. (2003) Preparation and characterization of folate-targeted pEG-coated pDMAEMA-based polyplexes. *J. Controlled Release* 87, 167–176.

(28) Bennis, J. M., Maheshwari, A., Furgeson, D. Y., Mahato, R. I., and Kim, S. W. (2001) Folate-PEG-folate-graft-polyethylenimine-based gene delivery. *J. Drug Target.* 9, 123–139.

- (29) Neradovic, D., van Nostrum, C. F., and Hennink, W. E. (2001) Thermoresponsive polymeric micelles with controlled instability based on hydrolytically sensitive N-isopropylacrylamide copolymers. *Macromolecules* 34, 7589–7591.
- (30) Talelli, M., Rijcken, C. J. F., Oliveira, S., van der Meel, R., van Bergen en Henegouwen, P. M. P., Lammers, T., van Nostrum, C. F., Storm, G., and Hennink, W. E. (2011) Nanobody – Shell functionalized thermosensitive core-crosslinked polymeric micelles for active drug targeting. *J. Controlled Release* 151, 183–192.
- (31) Soga, O., van Nostrum, C. F., Ramzi, A., Visser, T., Soulimani, F., Frederik, P. M., Bomans, P. H. H., and Hennink, W. E. (2004) Physicochemical characterization of degradable thermosensitive polymeric micelles. *Langmuir* 20, 9388–9395.
- (32) Jiang, X., van der Horst, A., van Steenbergen, M., Akeroyd, N., van Nostrum, C., Schoenmakers, P., and Hennink, W. (2006) Molar-mass characterization of cationic polymers for gene delivery by aqueous size-exclusion chromatography. *Pharm. Res.* 23, 595–603.
- (33) Grasseti, D. R., and Murray, J. F. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (34) Li, Y.-P., Pei, Y.-Y., Zhang, X.-Y., Gu, Z.-H., Zhou, Z.-H., Yuan, W.-F., Zhou, J.-J., Zhu, J.-H., and Gao, X.-J. (2001) PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. *J. Controlled Release* 71, 203–211.
- (35) Zugates, G. T., Anderson, D. G., Little, S. R., Lawhorn, I. E. B., and Langer, R. (2006) Synthesis of poly(β -amino ester)s with thiol-reactive side chains for DNA delivery. *J. Am. Chem. Soc.* 128, 12726–12734.
- (36) Ryu, J.-H., Chacko, R. T., Jiwanich, S., Bickerton, S., Babu, R. P., and Thayumanavan, S. (2010) Self-cross-linked polymer nanogels: A versatile nanoscopic drug delivery platform. *J. Am. Chem. Soc.* 132, 17227–17235.
- (37) Kameyama, S., Horie, M., Kikuchi, T., Omura, T., Tadokoro, A., Takeuchi, T., Nakase, I., Sugiura, Y., and Futaki, S. (2007) Acid wash in determining cellular uptake of Fab/cell-permeating peptide conjugates. *Biopolymers* 88, 98–107.
- (38) van Gaal, E. V. B., van Eijk, R., Oosting, R. S., Kok, R. J., Hennink, W. E., Crommelin, D. J. A., and Mastrobattista, E. (2011) How to screen non-viral gene delivery systems in vitro? *J. Controlled Release* 154, 218–232.
- (39) Funhoff, A., van Nostrum, C., Janssen, A., Fens, M., Crommelin, D., and Hennink, W. (2004) Polymer side-chain degradation as a tool to control the destabilization of polyplexes. *Pharm. Res.* 21, 170–176.
- (40) Luten, J., van Steenbergen, M. J., Lok, M. C., de Graaff, A. M., van Nostrum, C. F., Talsma, H., and Hennink, W. E. (2008) Degradable PEG-folate coated poly(DMAEA-co-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression. *Eur. J. Pharm. Sci.* 33, 241–251.
- (41) Campbell, I. G., Jones, T. A., Foulkes, W. D., and Trowsdale, J. (1991) Folate-binding protein is a marker for ovarian cancer. *Cancer Res.* 51, 5329–38.
- (42) Mislick, K. A., Baldeschwieler, J. D., Kayyem, J. F., and Meade, T. J. (1995) Transfection of folate-polylysine DNA complexes: Evidence for lysosomal delivery. *Bioconjugate Chem.* 6, 512–515.
- (43) Zhao, X., Li, H., and Lee, R. J. (2008) Targeted drug delivery via folate receptors. *Expert Opin. Drug Delivery* 5, 309–19.
- (44) Aa, M. A. E. M., Huth, U. S., Häfele, S. Y., Schubert, R., Oosting, R. S., Mastrobattista, E., Hennink, W. E., Peschka-Süss, R., Koning, G. A., and Crommelin, D. J. A. (2007) Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. *Pharm. Res.* 24, 1590–1598.
- (45) Yang, Z., Sahay, G., Sriadibhatla, S., and Kabanov, A. V. (2008) Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. *Bioconjugate Chem.* 19, 1987–1994.
- (46) Vercauteren, D., Rejman, J., Martens, T. F., Demeester, J., De Smedt, S. C., and Braeckmans, K. (2012) On the cellular processing of non-viral nanomedicines for nucleic acid delivery: Mechanisms and methods. *J. Controlled Release* 161, 566–581.
- (47) Wolfert, M. A., and Seymour, L. W. (1998) Chloroquine and amphipathic peptide helices show synergistic transfection in vitro. *Gene Ther.* 5, 409–414.
- (48) Liu, L., Zheng, M., Renette, T., and Kissel, T. (2012) Modular synthesis of folate conjugated ternary copolymers: polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol)-folate for targeted gene delivery. *Bioconjugate Chem.* 23, 1211–1220.
- (49) Dohmen, C., Fröhlich, T., Lachelt, U., Rohl, I., Vormlocher, H.-P., Hadwiger, P., and Wagner, E. (2012) Defined folate-PEG-siRNA conjugates for receptor-specific gene silencing. *Mol. Ther. Nucleic Acids* 1, e7.
- (50) Zhao, F., Yin, H., Zhang, Z., and Li, J. (2013) Folic acid modified cationic γ -cyclodextrin-oligoethylenimine star polymer with bioreducible disulfide linker for efficient targeted gene delivery. *Biomacromolecules* 14, 476–484.
- (51) Salvati, A., Pitek, A. S., Monopoli, M. P., Prapainop, K., Bombelli, F. B., Hristov, D. R., Kelly, P. M., Aberg, C., Mahon, E., and Dawson, K. A. (2013) Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface. *Nat. Nanotechnol.* 8, 137–143.