molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

Targeted Decationized Polyplexes for siRNA Delivery

Luís Novo,[†] Kaori M. Takeda,[‡] Tamara Petteta,[†] George R. Dakwar,[§] Joep B. van den Dikkenberg,[†] Katrien Remaut,[§] Kevin Braeckmans,[§,∥] Cornelus F. van Nostrum,[†] Enrico Mastrobattista,[†] and Wim E. Hennink*,[†]

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

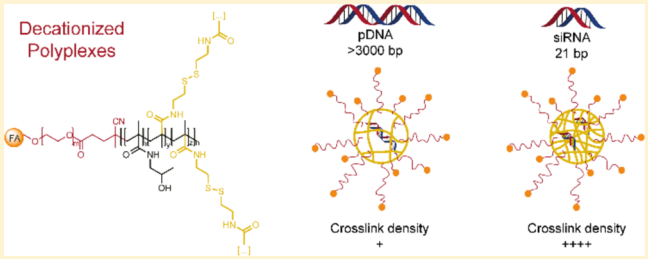
[‡]Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

[§]Laboratory for General Biochemistry and Physical Pharmacy, Faculty of Pharmacy, Ghent University, Ghent Research Group on Nanomedicines, Harelbekestraat 72, 9000 Ghent, Belgium

[∥]Centre for Nano- and Biophotonics, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

Supporting Information

ABSTRACT: The applicability of small interfering RNA (siRNA) in future therapies depends on the availability of safe and efficient carrier systems. Ideally, siRNA delivery requires a system that is stable in the circulation but upon specific uptake into target cells can rapidly release its cargo into the cytoplasm. Previously, we evaluated a novel generation of carrier systems ("decationized" polyplexes) for DNA delivery, and it was shown that folate targeted decationized polyplexes had an excellent safety profile and showed intracellular triggered release upon cell specific uptake.



Targeted decationized polyplexes consist of a core of disulfide cross-linked poly(hydroxypropyl methacrylamide) (pHPMA) stably entrapping nucleic acids and a shell of poly(ethylene glycol) (PEG) decorated with folate molecules. In the present study, the applicability of folate targeted decationized polyplexes for siRNA delivery was investigated. This required optimization of the carrier system particularly regarding the cross-linking density of the core of the polyplexes. Stable and nanosized siRNA decationized polyplexes were successfully prepared by optimizing the cross-link density of their core. Upon incubation in human plasma, a significant portion of siRNA remained entrapped in the decationized polyplexes as determined by fluorescence correlation spectroscopy (FCS). When tested in a folate receptor overexpressing cell line stably expressing luciferase, Skov3-luc, sequence specific gene silencing was observed. As expected, neither interference on the intrinsic luciferase expression nor on the cell metabolic activity (determined by XTT) was induced by the free-polymer or the siRNA polyplexes. In conclusion, targeted decationized polyplexes are safe and stable carriers that interact with the targeted cells and rapidly disassemble upon cell entry making them promising siRNA delivery systems.

KEYWORDS: *siRNA delivery, polymer, targeting, nanoparticle, biocompatibility*

INTRODUCTION

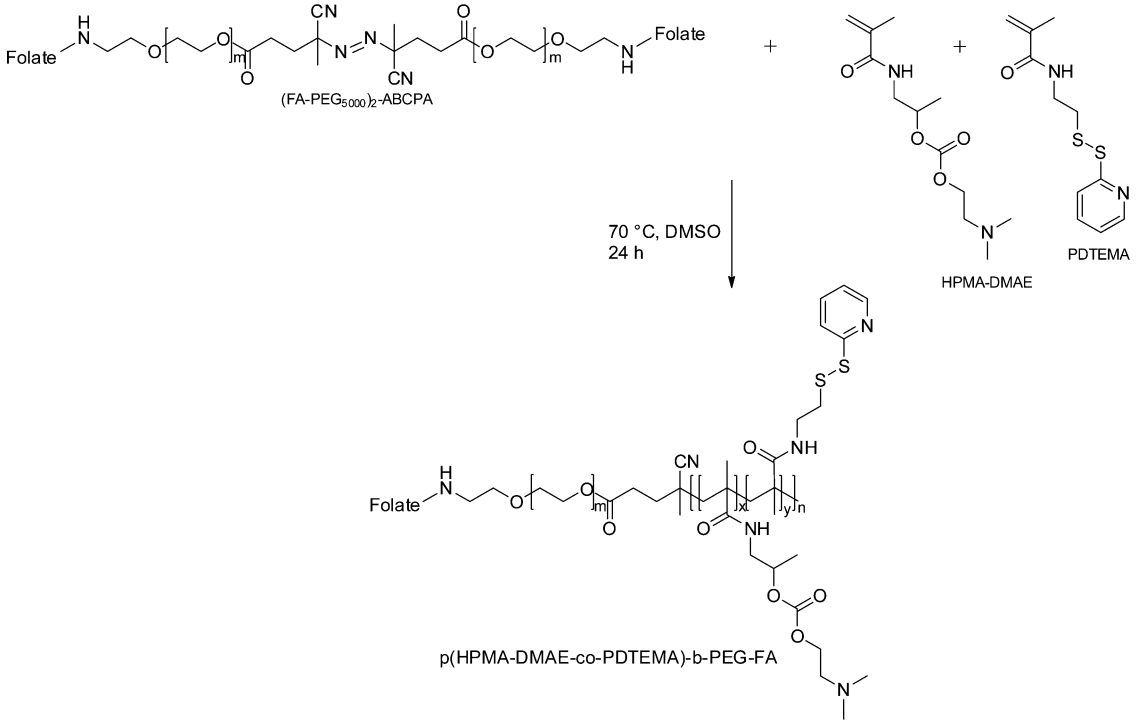
The use of small interfering RNA (siRNA) is currently explored as gene therapy strategy to treat several diseases, including viral infections, neurodegenerative disorders, and cancer.[1,2] siRNA therapies are based on the ability to induce efficient and specific gene silencing to reduce or eliminate expression of any target protein of interest. siRNA is composed of a 20−23 double strand nucleic acid sequence. siRNA can be synthetically produced and, when introduced in the cytoplasm of the cells, it is incorporated into a protein complex called the RNA-induced silencing complex (RISC). The siRNA activated RISC catalyzes subsequently the degradation of mRNA strands complementary to siRNA, blocking its translation into target proteins.[3,4]

siRNA is especially interesting because, upon release into the cytoplasm, transport into the nucleus is not necessary to exert its pharmacological action, unlike therapeutic DNA. However, just like DNA, siRNA cannot penetrate cellular membranes by passive diffusion due to its high molecular weight and hydrophilic character. Furthermore, siRNA can be easily degraded by nucleases present in the bloodstream. Therefore, systemic administration of siRNA requires safe and efficient carrier systems.

Recently, several siRNA delivery systems based on polymers, lipids, peptides, or proteins have been developed.[4−6] Cationic polymers form nanoparticles with siRNA via electrostatic

Received: July 23, 2014
Revised: October 14, 2014
Accepted: November 10, 2014
Published: November 10, 2014

Scheme 1. Synthesis of p(HPMA-DMAE-*co*-PDTEMA)-b-PEG by Free-Radical Polymerization of HPMA-DMAE, PDTEMA, and Using $(FA\text{-}PEG_{5000})_2$-ABCPA Macroinitiator



interactions, referred to as "polyplexes". It has been observed in many studies that polyplexes require modification of the carrier surface with a neutral and hydrophilic polymer [e.g., poly(ethylene glycol) (PEG)] to shield their charge and minimize unwanted interactions with constituents present in extracellular fluids. Furthermore, PEGylation results in polyplexes with higher colloidal stability.[7,8] The surface decoration of polyplexes with PEG, however, avoids neither unfavorable biodistribution to healthy (nontarget) organs, particularly liver, spleen, and kidneys nor premature dissociation of polyplexes resulting in release of free siRNA in the circulation.[9−13]

Because of the items mentioned above, efficient in vivo delivery of siRNA requires a system that is highly stable in the circulation and extracellular milieu, while retaining the ability to readily dissociate and release the loaded siRNA within the cell,[14−16] which can be achieved by introduction of disulfide cross-links in the core of siRNA polyplexes.[17,18] Disulfide cross-linking of nanocarriers is particularly interesting because these linkages are stable in the bloodstream but prone to rapid cleavage in reducing conditions such as the intracellular environment.[19]

Besides the severe effects induced at the systemic level (e.g., embolism or blood coagulation), the cationic nature of conventional polyplexes leads to severe toxicity at the cellular level as well.[4,20−22] So far, significant improvements have been reported regarding avoidance of systemic toxicity of polyplexes by PEgylation, however, cellular toxicity of polycations can only be partially reduced (i.e., by the use of biodegradable polymers).[23,24] Polycations can affect the cell viability at many levels, since they compromise the cell membrane integrity,[25−27] interact with crucial cellular polyanions (i.e., cell receptors, enzymes, mRNA, or genomic DNA),[28] interfere with cell expression profile,[29−31] and activate oncogenes or induce apoptosis.[26,31,32]

Given the insufficient in vivo stability of siRNA-polyplexes as well as their poor biodistribution and their intrinsic toxicity, alternatives for cationic polymers to design polyplexes are urgently needed. Decationized polyplexes, based on non-charged and hydrophilic polymers, have been developed as interesting plasmid DNA (pDNA) gene delivery systems.[33] These polyplexes consist of a core of disulfide cross-linked poly(hydroxypropyl methacrylamide) (pHPMA), in which pDNA is entrapped, and a PEG shell. The formation and encapsulation of negatively charged nucleic acid molecules is first driven by electrostatic interactions using the presence of cationic charges in the polymer. After complex formation, followed by structure stabilization exploiting disulfide cross-linking, decationization occurs by hydrolytic cleavage of the carbonate ester groups which connect the cationic side groups to the pHPMA backbone. These decationized polyplexes showed a high and stable DNA loading, which is exclusively based on physical entrapment of the nucleic acid in the disulfide cross-linked core. Therefore, decationized polyplexes are intracellularly destabilized, resulting in release of their payload by the specific cleavage of disulfide cross-links in the reducing environment of the cytosol. Decationized polyplexes are based on neutral polymers which explains their excellent cytocompatibility and their low teratogenicity and low mortality potential in a zebrafish toxicity assay.[33,34] These particles also have shown a low degree of unspecific uptake, together with a very high degree of cell specific uptake and transfection upon introduction of a targeting ligand at the distal end of the PEG shell.[35] The preparation of folic acid (FA) targeted decationized polyplexes is particularly interesting because this small targeting molecule selectively binds with high affinity to the folate receptor, which is specifically overexpressed in several tumor types, including metastatic forms.[36] Consequently, folate decorated nanomedicines have been developed for tumor targeting.[37] FA targeting has also demonstrated its feasibility for

dx.doi.org/10.1021/mp500499x | *Mol. Pharmaceutics* 2015, 12, 150−161

siRNA delivery to improve the specificity of gene silencing.[38−40]

In the present study, the applicability of folate targeted decationized polyplexes siRNA loading and release has been investigated. The applicability of decationized polyplexes for formulation and delivery of siRNA required optimization of both the polymer and the particle preparation process. Given its size, <1/100 the length of plasmid DNA, and stiff backbone structure, siRNA has an inherently poor binding affinity for polycations, and consequently, siRNA complexation and retention by cationic polymers is more challenging than that for pDNA.[41] In the present study, we describe the optimization steps for the preparation of siRNA-loaded decationized polyplexes, as well as their stability and in vitro cytocompatibility and gene silencing activity.

MATERIALS AND METHODS

Materials. 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.[33] Agarose multipurpose was purchased from Roche Molecular Biochemicals (Mannheim, Germany). 6× DNA Loading Dye was purchased from Fermentas (St. Leon-Roth, Germany). SYBR Safe DNA gel stain, Lipofectamine 2000, folate free RPMI-1640 medium, Opti-MEM, and dialyzed fetal bovine serum (FBS) were purchased from Life Technologies (Breda, The Netherlands). The Skov3-luc (human ovarian carcinoma cell line stably expressing firefly luciferase) cell line was obtained from Cell BioLabs (San Diego). Luciferase assay kit was obtained from Promega (Leiden, The Netherlands). All other chemicals, reagents, and media were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

The following buffer systems were used: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for buffering at pH 6.8−8.2; 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]-amino]propane-1-sulfonic acid (TAPS) for buffering solutions at pH 7.7−9.1; phosphate buffered saline, pH 7.4 (PBS) [obtained from B. Braun (Melsungen, Germany)].

The siRNA sequences were used as follows: luciferase GL3 siRNA (anti-Luc siRNA, directed against luciferase) (ThermoScientific, Etten-Leur, The Netherlands), sense strand 5′-CUU ACG CUG AGU ACU UCG AdTdT-3′; negative control LV2 (scramble siRNA), sense strand 5′-AUC GUA CGU ACC GUC GUA UdTdT-3′; Alexa 647 labeled negative control LV2 at the 3′ end of the sense strand [kindly donated by Dr. Mastrobattista (Dept. Pharmaceutics, UU)].

Polymer Synthesis. *Synthesis of (FA-PEG)$_2$-ABCPA.* The synthesis and characterization of folate-PEG (M_w 5000 Da) bifunctionalized azo-macronitiator [(FA-PEG)$_2$-ABCPA] was performed as previously described.[35]

Synthesis of pHDP-PEG-FA. p(HPMA-DMAE-*co*-PDTEMA)-*b*-PEG-FA (pHDP-PEG-FA) was synthesized by free radical polymerization using (FA-PEG)$_2$-ABCPA as macroinitiator (Scheme 1). The polymer was synthesized using a HPMA-DMAE-to-initiator ratio (M/I) of 150 (mol/mol) and a feed ratio HPMA-DMAE/PDTEMA of 1/0.6 (mol/mol). The polymerization was carried at 70 °C for 24 h in DMSO under a N_2 atmosphere, using a 2.5 μmol macroinitiator and monomer concentration of 0.8 M. After polymerization, the product was precipitated in diethyl ether and collected by centrifugation, dialyzed against 5 mM NH_4Ac buffer pH 5.0 for 3 days at 4 °C (MWCO 6000−8000), and collected by freeze-drying. Unreacted FA-PEG present in the product was removed by precipitation in cold EtOH (5 mg/mL of solids) followed centrifugation. The pHDP-PEG-FA soluble in the EtOH supernatant was collected after EtOH evaporation, dissolution in water, and freeze-drying.

Preparation of p(HPMA-co-PDTEMA)-b-PEG-FA. p(HPMA-*co*-PDTEMA)-*b*-PEG-FA (pHP-PEG-FA) was prepared by dissolving 5 mg of pHDP-PEG-FA in 2.5 mL of 10 mM HEPES, 10 mM TAPS, pH 8.5, and incubated at 37 °C for 6 h to hydrolyze the carbonate ester bonds linking the dimethylaminoethanol (DMAE) side groups to the polymer backbone.[33] After hydrolysis, the polymer was purified with a PD-10 desalting column following the supplier's protocol and collected by freeze-drying.

Polymer Characterization. *Gel Permeation Chromatography (GPC) Characterization of the Polymers.* Analysis of the (FA-PEG)$_2$-ABCPA macroinitiator and pHDP-PEG-FA polymer was performed using a Waters System (Waters Associates Inc., Milford, MA) with refractive index (RI) and UV detection using two serial Plgel 5 μm MIXED-D columns (Polymer Laboratories) and DMF containing 10 mM LiCl as eluent. The flow rate was 1 mL/min and the temperature was 60 °C. UV detection of FA groups was done at 363 nm. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity (PDI, M_w/M_n) of pHDP PEG-FA was determined using a series of PEG calibration standards.

^{1}H NMR Characterization of the Polymers. The copolymer composition was determined by ^{1}H NMR. The NMR spectrum of the copolymer dissolved in DMSO-*d*6 was recorded on a 400 MHz Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara). The copolymer composition and calculation of the number-average molecular weight (M_n) was performed as previously described.[33] The ratio HPMA-DMAE/PDTEMA was determined by comparison of the integrals at δ4.6 ppm (bs, $CH_2C\underline{H}CH_3O$, HPMA-DMAE) and the integral at δ8.5 ppm (bs, pyridyl group proton, PDTEMA) ($\int\delta 4.6/\int\delta 8.5$).

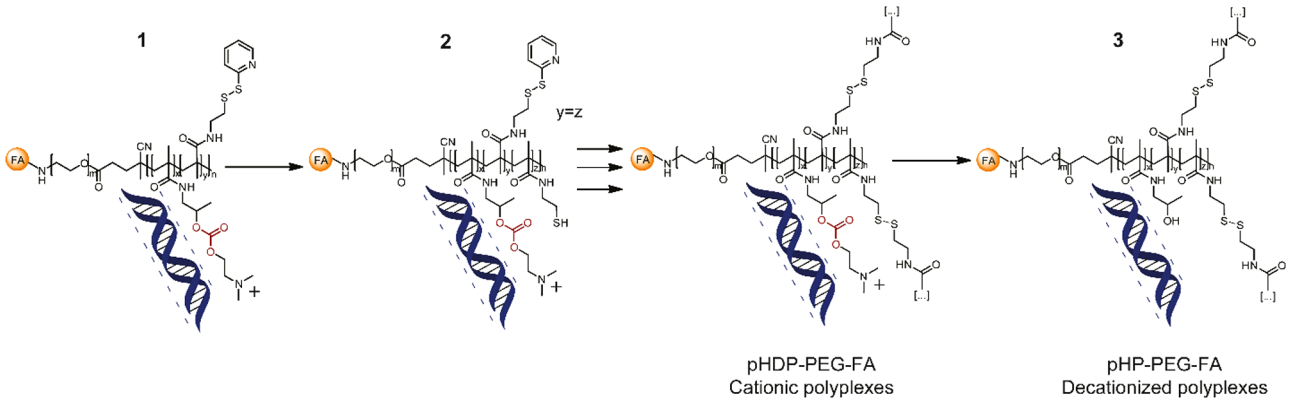
Number-average molecular weight (M_n) of the polymer was determined according to eq 1:

$$M_n = \left(\int\delta 4.6 \times M_{\text{HPMA-DMAE}} + \int\delta 8.5 \times M_{\text{PDTEMA}}\right) / \left(\int\delta 3.5/448\right) + M_{\text{FA-PEG5000}}\ (\text{g/mol}) \quad (1)$$

where, $\int\delta 3.5$, $\int\delta 4.6$, and $\int\delta 8.5$ are the integrals at 3.5, 4.6, and 8.5 ppm, respectively. $M_{\text{HPMA-DMAE}}$ and M_{PDTEMA} are the molar masses of HPMA-DMAE and PDTEMA, respectively. The molar mass of FA-PEG$_{5000}$ block ($M_{\text{FA-PEG5000}}$) was set to 5441 g/mol. The number of protons for the FA-PEG$_{5000}$ block, this is $\int\delta 3.5$, was set to 448.

UV Spectroscopy Characterization. The quantification of the molarity of thiol reactive pyridyl disulfide (PDS) groups per weight of polymer was performed by UV spectroscopy on a Shimadzu UV-2450 UV/vis spectrophotometer ('s-Hertogenbosch, The Netherlands). pHDP-PEG-FA stock solutions of 1 mg/mL in 20 mM HEPES, pH 7.4, containing 50 mM tris(2-carboxyethyl)phosphine (TCEP) were prepared, and after incubation at 37 °C for 1 h, the UV absorbance at 343 nm was measured to determine the release of 2-mercaptopyridine.[42] Quantification was performed using a calibration curve with 2-mercaptopyridine standards.

Scheme 2. Three-Step Preparation of Interchain Disulfide-Crosslinked Decationized Polyplexes: (1) Charge-Driven Condensation with Nuclei Acids; (2) Stabilization through Disulfide Crosslinking by Addition of Half Molar Equivalents of DTT to PDS Groups in the Polymer; (3) Decationization of Cationic pHDP-PEG-FA Polyplexes at 37°C for 6 h, pH 8.5, Resulting in Decationized pHP-PEG-FA Polyplexes (Adapted from Novo et al.[33])



Preparation of Decationized Polyplexes. The method previously described for the preparation of pDNA decationized polyplexes[33] was optimized for the entrapment of siRNA (Scheme 2). The amount of polymer added to complex siRNA was optimized on the basis of the *N*/*P* ratio [*N*, molarity protonable amines from polymer; *P*, molarity of negatively charged phosphates siRNA)]. Polymer solutions of pHDP-PEG-FA (e.g., for *N*/*P* = 8, 1 mg/mL polymer solution, 105 μL) were mixed with 210 μL siRNA (concentration 7500 nM) in 10 mM HEPES and 10 mM TAPS, pH 8.5, by vortexing.

After complexation, the polyplexes were cross-linked for 1 h at pH 8.5 by addition of a half molar equivalent of dithiothreitol (DTT) to PDS groups of the polymer, in order to induce self-cross-linking of the polyplexes.[43] Alternatively, cross-linking was performed by addition of the dithiol 3,6-dioxa-1,8-octanedithiol (DODT) corresponding with a molar equivalent of DODT thiol groups to PDS groups of the polymer.

After cross-linking, the cationic DMAE side groups were removed by hydrolysis by incubation of the polyplex dispersions at 37 °C for 6 h and pH 8.5, to yield pHP-PEG-FA polyplexes.[33] Next, the pH of the dispersion was adjusted to pH 7.4 with 1 M HCl, and the ionic strength was adjusted to 150 mM with 1.5 M NaCl, diluting the polyplexes to a final siRNA concentration of 4000 nM. Depending on the application, the polyplex dispersions were further diluted in PBS (10 mM Na_2HPO_4/NaH_2PO_4, 140 mM NaCl, pH 7.4) or 20 mM HEPES, pH 7.4. For comparative studies, siRNA cationic pHDP-PEG-FA polyplexes were prepared by adjusting the pH to 7.4 with 1 M HCl immediately after cross-linking.

For in vitro testing, the polyplex dispersions were purified by dialysis overnight against sterile PBS using Slide-A-Lyzer MINI Dialysis Devices 20K MWCO (Thermo Fisher Scientific).

Particle Size and Zeta Potential Determination. *Dynamic Light Scattering (DLS).* The size of the polyplexes was measured with DLS on an ALV CGS-3 system (Malvern Instruments, Malvern, U.K.) equipped with a JDS Uniphase 22 mW He−Ne laser operating at 632.8 nm, an optical fiber-based detector, and a digital LV/LSE-5003 correlator with temperature controller set at 25 or 37 °C. Measurements were performed in PBS at a final siRNA concentration of 500 nM.

Zeta Potential. The zeta potential of the polyplexes was measured using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, U.K.) with universal ZEN 1002 "dip" cells and DTS (Nano) software (version 4.20) at 25 °C. Zeta potential measurements were performed on polyplex dispersions in 20 mM HEPES, pH 7.4, at a siRNA concentration of 500 nM.

Nanoparticle Tracking Analysis (NTA). Size distribution of the polyplexes was determined by NTA on a NanoSight LM10SH (NanoSight, Amesbury, United Kingdom), equipped with a sample chamber with a 532 nm laser. Using a siRNA polyplex concentration of 1 nM in PBS, videos of 160 s were taken and analyzed by NTA 2.0 image analysis software (NanoSight, Amesbury, U.K.). The detection threshold was set at 2 and the minimum track length at 10. The mode and mean size and SD values were reported by the NTA software.

Transmission Electron Microscopy (TEM). The size and morphology of the polyplexes were analyzed using transmission electron microscopy (TEM, FEI Tecnai T10). A droplet of polyplex dispersion of 1000 nM siRNA in a phosphate-free and low ionic strength buffer (20 mM HEPES pH 7.4) was placed on a carbon-coated copper grid. The samples were stained with 2% uranyl acetate. Size determination was done with Olympus MeasureIT software.

Gel Retardation Assay. Decationized pHP-PEG-FA and cationic pHDP-PEG-FA polyplexes were incubated at 37 °C for 2.5 h in the absence or presence of 10 mM DTT (as a reducing agent). Next, 15 μL (1000 nmol siRNA) of polyplex dispersion in PBS was mixed with 3 μL 6× DNA loading dye, and the polyplexes were loaded into 2% agarose gel in a tris-acetate-EDTA (TAE) buffer stained with SYBR safe and run at 120 V for 30 min. The gel was analyzed by a Gel Doc XR+ system (BioRad Laboratories Inc., Hercules, CA) with Image Lab software.

Polyplex Stability Determined by Fluorescence Correlation Spectroscopy (FCS). siRNA release from the polyplexes during 2 h of incubation with 20 mM HEPES buffer, pH 7.4, or in 90% human plasma following incubation at 37 °C, was determined by FCS as previously described by Buyens et al.[44] Briefly, FCS measurements were performed on free Alexa 647-siRNA (λ_{exc} = 594 nm and λ_{em} = 633 nm) and Alexa 647-siRNA encapsulated in decationized pHP-PEG-FA polyplexes, on a C1si laser scanning confocal microscope (Nikon, Japan), equipped with a time-correlated single photon counting (TCSPC) data acquisition module (Picoquant, Berlin, Germany), and water immersion objective lens (Plan Apo 60×, NA 1.2, collar rim correction, Nikon, Japan). During the measurements, the glass bottom 96-well plate (Grainer Bio-

dx.doi.org/10.1021/mp500499x | *Mol. Pharmaceutics* 2015, 12, 150−161

one, Frickenhausen, Germany) was covered with adhesive plates seals (ThermoScientific, U.K.) to avoid evaporation of water. For each sample, fluorescence intensity fluctuations were recorded using Symphotime (Picoquant, Berlin, Germany), during 1 min in triplicate. As the baseline fluorescence intensity of the fluorescence fluctuation profiles recorded by FCS is proportional to the concentration of free siRNA, the percentage of complexed and released siRNA can be calculated as described and validated by Buyens et al.[44]

Cell Culture. Skov3-luc cells (folate receptor overexpressing cell line) were cultured in DMEM (4500 mg/L glucose) supplemented with antibiotics/antimycotics (penicillin, streptomycin sulfate, and amphotericin B) and 10% FBS (Sigma-Aldrich). Cells were maintained at 37 °C in a 5% CO_2 humidified air atmosphere.

Gene Silencing. Skov3-luc cells were seeded into 96-well plates (6000 cells per well). After 24 h, the DMEM medium was removed and replaced with folate-free RPMI 1640 supplemented with 10% dialyzed FBS, with or without 1 mM folic acid, containing siRNA polyplex dispersions at siRNA concentrations of 100, 250, 500, and 1000 nM (anti-Luc and scramble siRNA). The cells were incubated with siRNA polyplexes at 37 °C for 24 h. Lipofectamine 2000 (Life Technologies) siRNA complexes were prepared according to the manufacturer's protocol and used as positive control for transfection,[14] and PBS treated cells were used as a negative control. Alternatively, pHP-PEG-FA free polymer (concentrations ranging from 0.001 to 1 mg/mL) was also incubated with the cells for 24 h.

Luciferase cellular expression was measured 48 h after transfection. Cells were washed with 100 μL of cold PBS and lysed with 50 μL of lysis buffer [reporter lysis buffer 5× (Promega), diluted in mQ H_2O]. Cell lysis was completed by performing a freeze−thaw cycle after placing the cells at −80 °C for 1 h. Next, 20 μL of cell lysate was mixed with 100 μL of luciferase assay reagent (Promega), and 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 is expressed as relative light units (RLU).

Toxicity. The effect of the decationized pHP-PEG-FA in soluble form or pHP-PEG-FA siRNA polyplexes on the cell metabolic was determined by the XTT assay. Free polymer (concentrations ranging from 0.001 to 1 mg/mL) or polyplexes (concentrations ranging from 100 to 1000 nM siRNA) were incubated with the Skov3-luc cells in 96-well plates (6000 cells per well) for 24 h with folate-free RPMI 1640 supplemented with 10% dialyzed FBS. XTT assay was performed 48 h after incubation of the polymer or polyplexes with the cells.

The XTT assay was performed by incubating Skov3-luc cells at 37 °C for 1 h in a CO_2-incubator after addition of 50 μL per well of a freshly prepared XTT solution (25 μM *N*-methyl dibenzopyrazine methylsulfate (PMS) and 1 mg/mL 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in plain RPMI 1640 medium). The relative cell metabolic activity was calculated by normalizing the absorbance at 490 nm (reference wavelength of 655 nm) with the absorbance of buffer-treated cells.

Statistical Analysis. Statistical analyses were performed with the software GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California). A two-tailed paired Student's *t* test was used, where $p < 0.05$ was considered to represent statistical significance.

RESULTS AND DISCUSSION

Polymer Synthesis. FA functionalized polymer for the preparation of targeted decationized polyplexes was synthesized by free radical copolymerization of HPMA-DMAE with PDTEMA using the FA-PEG_{5000} bifunctionalized $(FA\text{-}PEG)_2$-ABCPA as macroinitiator (Scheme 1). The polymerization resulted in the formation of the block copolymer p(HPMA-DMAE-*co*-PDTEMA)-*b*-PEG-FA with a yield close to 50%. The characteristics of the synthesized polymer were established by ^{1}H NMR, GPC, and UV spectroscopic analysis and are given in Table 1. Although both HPMA-DMAE and PDTEMA are methacrylamide monomers, incorporation of PDTEMA was slightly lower than the feed composition, as previously observed.[33] The M_n calculated from ^{1}H NMR spectroscopic analysis (34.9 kDa) was close to that determined with GPC (39.7 kDa, PDI = 2.1). UV spectroscopy was used to determine the incorporation of PDTEMA (630 ± 21 nmol PDTEMA per milligram of polymer).

Table 1. p(HPMA-DMAE-co-PDTEMA)-b-PEG-FA Characteristics Determined by GPC, ^{1}H NMR, UV Spectroscopic Analysis

GPC	NMR				UV
M_n (kDa)	PDI	M_n (kDa)	feed HPMA-DMAE/PDTEMA	copolymer HPMA-DMAE/PDTEMA	$nmol_{PDS}/mg_{polymer}$
39.7	2.1	34.9	1.7/1	2.5/1	630 ± 21

The cationic HPMA-DMAE monomer was incorporated in the copolymer to allow electrostatic interaction of the polymer with siRNA to yield polyplexes. The PDTEMA monomer contains the PDS functionality, which allows interchain disulfide cross-linking of the core of the polyplexes by the thiol−disulfide exchange reaction. The copolymer composition, in particular the HPMA-DMAE/PDTEMA molar ratio, ultimately determines the cross-link density of the core of the polyplexes. After cross-linking, the labile DMAE cationic groups can be removed by hydrolysis to yield the decationized polyplexes based on PEG-pHPMA.[45] Both PEG and pHPMA are frequently used in advanced drug delivery systems because of their good safety profile. Furthermore, chemical derivatization of the HPMA monomer with a DMAE group to yield HPMA−DMAE gives after polymerization the desired decationizable structure. PDTEMA is also a methacrylamide monomer and can be randomly copolymerized with HPMA-DMAE. Importantly, the polymers were designed to include a PEG block in order to shield and sterically stabilize the cross-linked pHPMA core of the polyplexes. PEG is an essential part of decationized polyplexes because this polymer improves the in vivo stability of nanoparticles, by avoiding aggregation and protein adsorption in blood, and it reduces the recognition of nanoparticles by the mononuclear phagocyte system. This is quite evident for cationic particles,[46−48] but it has also been found to be essential for neutral or anionic particles,[49−51] like the decationized polyplexes.

Preparation and Stability of Decationized pHP-PEG-FA Polyplexes Loaded with siRNA. siRNA Polyplexes based on pHP-PEG-FA were formed through a three-step process in a similar process as described for the preparation of pDNA polyplexes[33,35] (Scheme 2). The first step was the electrostatic complexation of the cationic block copolymer p(HPMA-DMAE-*co*-PDTEMA)-*b*-PEG-FA (pHDP-PEG-FA) with siRNA. The stabilization of the polyplexes was subsequently

established via interchain disulfide cross-linking using DTT. The addition of half molar equivalents of DTT to PDS groups of the polymer, induced self-cross-linking of the polyplexes[43] to yield disulfide cross-linked cationic pHDP-PEG-FA polyplexes. To further explain, DTT was added to the polyplex solution to reduce 50% of the PDS groups in the core of the polyplexes, and subsequently, the newly generated thiols react with the remaining 50% PDS groups via thiol−disulfide exchange reaction to form the interchain disulfide cross-links in the core of the polyplexes. p(HPMA-*co*-PDTEMA)-*b*-PEG-FA (pHP-PEG) decationized polyplexes were obtained by removal of the DMAE cationic side groups from cationic pHDP-PEG-FA polyplexes by hydrolysis of the carbonate ester bond linking the DMAE side groups to the HPMA backbone for 6 h at pH 8.5. The hydrolysis time of 6 h has been previously established[33] as the minimum time required to obtain extensive loss of DMAE cationic groups (>99%), while avoiding unwanted loss of PEG chains. It is aimed that after decationization, the loaded siRNA is retained in the pHPMA cross-linked core of the polyplexes which are surrounded by a PEG shell containing the FA targeting ligand.

Previously, decationized polyplexes have been optimized for entrapment of pDNA using pHDP-PEG with an HPMA-DMAE/PDTEMA ratio of 6.5/1.[33,35] Stabilization of the polyplexes via interchain disulfide cross-linking occurred using the short dithiol DODT by thiol−disulfide exchange reaction between the thiol groups from DODT and PDS groups in the polymer. To fully complex siRNA and form stable polyplexes with pHDP-PEG-FA (HPMA-DMAE/PDTEMA 6.5/1) an N/P = 8 was used, and the ability of decationized pHP-PEG-FA polyplexes to retain the entrapped siRNA was evaluated by a gel retardation assay (Figure 1). Decationized polyplexes were also incubated with 10 mM DTT (lane 2) to mimic the intracellular reducing environment.[19,52] Retained siRNA in the polyplexes is visible in the starting slot of the well, whereas free or released siRNA migrates in the gel toward the anode. As shown in Figure 1, pHP-PEG-FA polyplexes which were prepared at an HPMA-DMAE/PDTEMA ratio of 6.5/1 were not able to stably entrap siRNA after loss of cationic DMAE groups, even in the absence of 10 mM DTT (lane 1). As a control, cross-linked pHDP-PEG-FA cationic polyplexes were also evaluated using the same assay, and it was shown that both in the absence (lane 1) and presence of 10 mM DTT (lane 2), no free siRNA was detected, which demonstrates that at N/P = 8 the formed cationic complexes quantitatively retained the added siRNA.

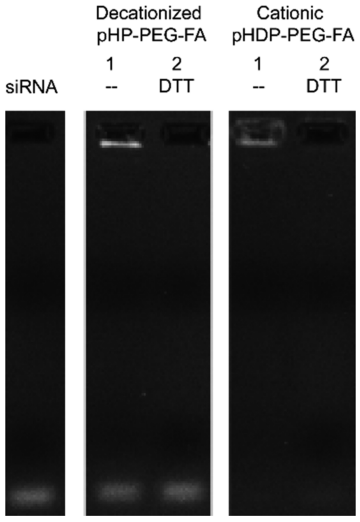


Figure 1. Agarose gel retardation assay of decationized pHP-PEG-FA and comparison of the cationic pHDP-PEG-FA polyplexes used as the control: lane 1, untreated sample; lane 2, sample treated with 10 mM DTT (control, free siRNA). Polyplexes were prepared using pHDP-PEG-FA with HPMA-DMAE/PDTEMA = 6.5/1, N/P = 8 and DODT as cross-linker.

These results point out the importance of considering the differences between pDNA and siRNA for optimization of siRNA decationized polyplexes. pDNA has a size between 3000 and 5000 bp, but siRNA is composed of only 21 bp and has a size of 8 nm (or <1/100 of pDNA length).[41,53] In addition, the rigidity of siRNA hampers its effective complexation with cationic polymers and adversely affects the stability of polyplexes.[54] Accordingly, effective retention of siRNA molecules in the core of decationized polyplexes required a more densely cross-linked core, when compared to pDNA-loaded decationized polyplexes. Therefore, in the present study, pHDP-PEG-FA with HPMA-DMAE/PDTEMA ratio of 2.5/1 was synthesized. A further increase of PDTEMA, a less hydrophilic monomer (HPMA-DMAE/PDTEMA 1.5/1), led to a reduced water solubility of the block copolymer. A good aqueous solubility is, however, essential for polyplex preparation, and this polymer was therefore not evaluated in this study.

DTT is a strong reducing agent that can be used as an alternative to the dithiol DODT and, unlike DODT, does not introduce any additional chemical structures in the newly formed crosslinks. Therefore, decationized polyplexes were prepared using pHDP-PEG-FA with a HPMA-DMAE/PDTEMA ratio of 2.5/1 and N/P = 8, using both DTT and DODT as cross-linking agents, and size distribution determination by NTA (Figure 2a) and gel retardation assay (Figure 2b) were performed. Size distribution of pHP-PEG-FA decationized polyplexes prepared with DODT as cross-linker showed a mean size of around 167 ± 63 nm, whereas polyplexes prepared with DTT possessed an evident smaller mean size of 99 ± 40 nm and a narrower size distribution (Figure 2a). When polyplexes prepared with both methods were evaluated with a gel retardation assay (Figure 2b), significant differences in the retention behavior of polyplexes were observed. In the case of decationized polyplexes cross-linked with DODT, siRNA was not fully encapsulated and a high concentration of free siRNA was detected (lane 1). In contrast, when decationized polyplexes were prepared using DTT as a cross-linking agent, complete siRNA retention in the polyplexes was observed (lane 2). The loss of electrostatic interactions between the polymer and siRNA leads to hydration and slight swelling of the polyplex core, when the short DODT cross-linker is used. However, by using DTT as a cross-linking agent, no additional structures are introduced between two cross-linking points (PDS groups). Consequently, swelling is minimized and a more cross-linked core with lower mesh size is obtained, preventing leakage of siRNA.

Decationized polyplexes are designed to stably entrap siRNA and simultaneously preserve the ability of intracellular triggered release. This latter property of decationized polyplexes using pHDP-PEG-FA with HPMA-DMAE/PDTEMA = 2.5/1, N/P = 8 and DTT as cross-linking agent was evaluated by a gel retardation assay (Figure 3) in the absence (lane 1) or presence of a reducing agent (10 mM DTT, lane 2). As demonstrated in Figure 3, decationized polyplexes completely entrapped siRNA

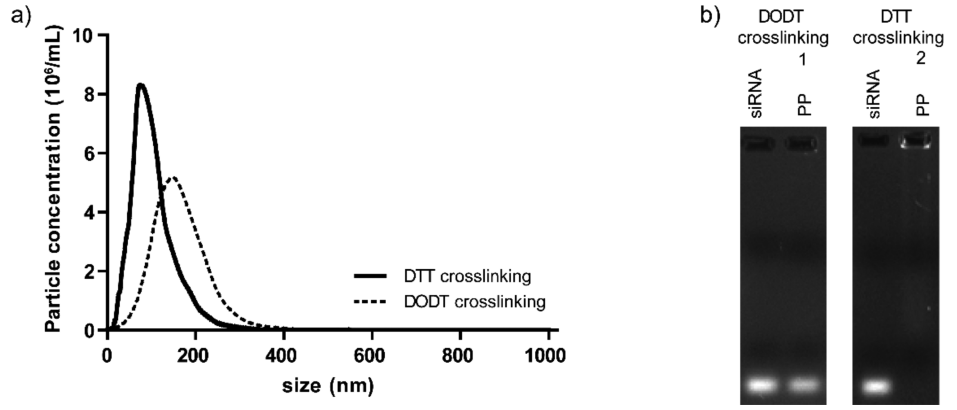


Figure 2. pHP-PEG-FA decationized polyplexes prepared using the dithiol DODT or DTT as cross-linking agents. (a) Polyplexes size distribution determined by NTA. (b) Agarose gel retardation assay: lane 1, DODT cross-linked polyplex (PP) untreated sample; lane 2, DTT cross-linked polyplex (PP) untreated sample (control, free siRNA). Polyplexes were prepared using pHDP-PEG-FA with HPMA-DMAE/PDTEMA = 2.5/1 and N/P = 8.

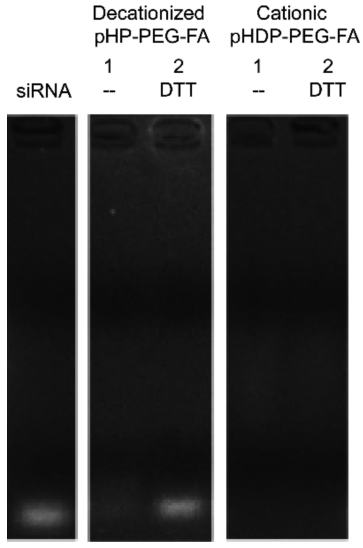


Figure 3. Agarose gel retardation assay of decationized pHP-PEG-FA and cationic pHDP-PEG-FA polyplexes: lane 1, untreated sample; lanes 2, sample treated with 10 mM DTT. Polyplexes were prepared using pHDP-PEG-FA with HPMA-DMAE/PDTEMA = 2.5/1, N/P = 8, and DTT to induce self-cross-linking.

when dispersed in PBS. However, when the polyplexes were incubated with 10 mM DTT for 2.5 h (lane 2) mimicking the intracellular reducing environment,[19,52] disulfide cross-links were cleaved, and the polyplexes completely released the encapsulated siRNA. As a control, cross-linked pHDP-PEG-FA cationic polyplexes were also studied, but no free siRNA was detected neither in the absence (lane 1) nor presence of DTT (lane 2) since siRNA was still retained in the polyplexes by electrostatic interactions between the negatively charged siRNA and the cationic polymer. These results demonstrate that siRNA encapsulation in pHP-PEG-FA decationized polyplexes is mainly due to physical entrapment. Moreover, in the presence of a reducing agent, polyplexes release their siRNA loading.

The polyplexes prepared with pHDP-PEG-FA with HPMA-DMAE/PDTEMA = 2.5/1, N/P = 8, and DTT as a cross-linking agent were further characterized and evaluated.

The particle size determined by DLS and zeta potential measurement results are shown in Table 2. The cationic pHDP-PEG-FA polyplexes formed at a N/P = 8 yielded nanosized particles with a diameter of 126 ± 2 nm and a positive zeta potential of +5.9 ± 0.4 mV. After decationization, pHP-PEG-FA polyplexes had a size of 123 ± 6 nm and a slightly negative zeta-potential of −2.6 ± 0.1 mV, which confirms the loss of the cationic DMAE groups[33] and retention of the negatively charged siRNA in the core. The similar sizes of both polyplexes proves that polyplex structure remains intact after decationization using a high cross-link density and the use of DTT as a cross-linking agent.

Table 2. Particle *z*-Average Diameter (Z-avg) and Polydispersity Index (PDI) Determined by DLS and Zeta Potential (ζ Pot) of Cationic pHDP-PEG-FA and Decationized pHP-PEG-FA Polyplexes[a]

	DLS		Zetasizer
polyplexes	*Z*-avg (nm)	PDI	ζ Pot (mV)
pHDP-PEG-FA	126 ± 2	0.14 ± 0.02	5.9 ± 0.4
pHP-PEG-FA	124 ± 2	0.12 ± 0.02	−2.6 ± 0.1

[a]Polyplexes were prepared with pHDP-PEG-FA with an HPMA-DMAE/PDTEMA ratio of 2.5/1 at N/P = 8. Results are expressed as mean ± SD (n = 3).

The size and shape of siRNA decationized pHP-PEG-FA polyplexes were also evaluated with TEM (Figure 4), and with this technique, small (∼30 nm) and spherical particles were

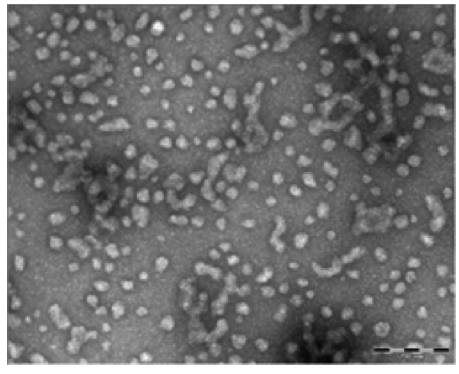


Figure 4. TEM images of decationized pHP-PEG-FA polyplexes prepared with pHDP-PEG-FA with a HPMA-DMAE/PDTEMA ratio of 2.5/1 at N/P = 8. Magnification 73000×. Scale bar = 200 nm.

detected. A small population of more elongated structures can also be observed which are probably the result of interparticle cross-linking. The discrepancy in size as measured by DLS and TEM analysis has been previously observed for PEGylated polyplexes and is most likely due to lack of uranyl acetate staining of the PEG corona.[48] Furthermore, the presence of a few larger particles in a sample has a great impact on the *z*-average size as measured by DLS, due to its sensitivity to large particles.[55]

The size of siRNA decationized pHP-PEG-FA polyplexes was also analyzed by NTA (Figure 5). NTA allows for

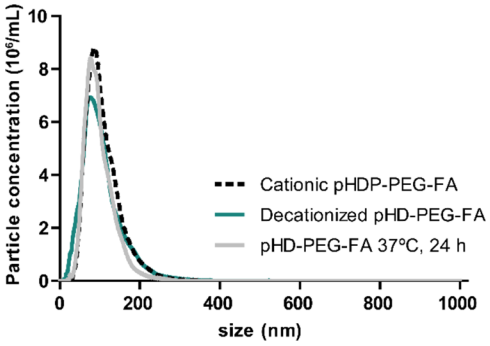


Figure 5. Cationic pHDP-PEG-FA and decationized pHP-PEG-FA siRNA (before and after incubation at 37 °C for 24 h in PBS) polyplexes size distribution determined by NTA. Polyplexes were prepared with pHDP-PEG-FA with a HPMA-DMAE/PDTEMA ratio of 2.5/1 at N/P = 8.

simultaneously analyses of a set of individual particles in a suspension and gives information on the real size distribution of the sample, which cannot be obtained with DLS.[55] The mean size and size distribution of decationized pHP-PEG-FA polyplexes was also compared with that of the cationic pHDP-PEG-FA polyplexes. In line with DLS data, the decationized and cationic polyplexes showed comparable average sizes (around 100 nm). The size distributions of both decationized and cationic polyplexes almost overlap (SD around 40 nm), demonstrating once more that the method to prepare siRNA decationized polyplexes using DTT as a cross-linking agent leads to efficient stabilization of the nanoparticles. The stability of decationized polyplexes at physiological ionic strength and pH was evaluated by incubation of the polyplexes in PBS at 37 °C for 24 h, followed by NTA analysis. It was shown that after incubation, decationized polyplexes maintained their size distribution as well as their particle count (Figure 5).

The stability of decationized pHP-PEG-FA polyplexes in physiological ionic strength and pH was further confirmed by continuous DLS measurement during incubation of the polyplexes at 37 °C for 24 h in PBS (Figure 6). This figure shows that the polyplexes maintained a stable size of around 120 nm as well as an almost constant intensity of scattered light for 24 h. These results show that decationized polyplexes were very stable without particle swelling, disassembling, or aggregation, demonstrating their excellent colloidal stability.

Stability of Decationized Polyplexes in Plasma. In vivo application of siRNA-loaded nanoparticles requires a high stability of the particles before reaching their target tissue. Often premature dissociation of siRNA from polyplexes occurs in the bloodstream or in the kidneys.[9−12] Thus, it is crucial to assess the stability of siRNA polyplexes in biological fluids to

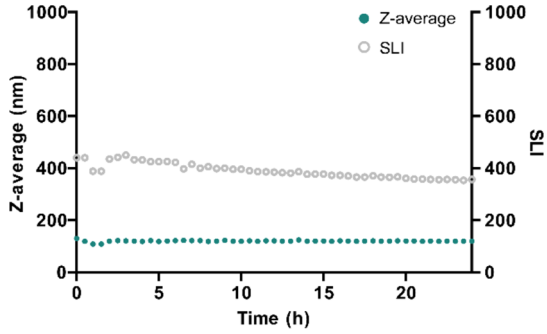


Figure 6. Particle size and scattered light intensity (SLI) of siRNA decationized pHP-PEG-FA polyplexes determined by DLS upon incubation in PBS at 37 °C for 24 h. Polyplexes were prepared with pHDP-PEG-FA with a HPMA-DMAE/PDTEMA ratio of 2.5/1 at N/P = 8.

get insight into the in vivo potential of these nanomedicines. FCS is an important technique to evaluate the stability of fluorophore-labeled siRNA complexes in undiluted biological fluids.[44,56] This technique is based on the measurement of fluorescence intensity fluctuations of labeled siRNA in the excitation volume of a confocal microscope. The average intensity of these fluctuations is proportional to free siRNA concentration. In the case of complexed siRNA, the average intensity is significantly decreased. The release of siRNA from polyplexes, will result in the presence of free siRNA in the sample, which can be determined by analysis of average intensity of the fluorescence.

Decationized pHP-PEG-FA polyplexes loaded with Alexa 647 labeled siRNA were incubated in human plasma, and the possible release of siRNA was studied with FCS for 2 h (Figure S1 of the Supporting Information). The amount of free siRNA was first evaluated in buffer, and analysis showed that less than 20% free siRNA in the polyplex dispersion was observed after decationization. When siRNA pHP-PEG-FA decationized polyplexes were incubated in plasma, a burst release of siRNA was detected (∼60%). However, after this initial release, the remaining siRNA remained entrapped in the polyplexes, up to 2 h. Most likely, siRNA molecules loosely entrapped in the polyplexes and located in the outer core of the polyplexes, dissociate from the polyplexes in the presence of plasma proteins. These siRNA molecules are probably kept in the polyplexes via weak hydrophobic interactions, in the compact core of siRNA polyplexes as previously observed for other polymer-siRNA complexes.[11,57] These interactions are broken in the presence of plasma proteins leading to release of siRNA from the polyplexes. Due to siRNA small size and inability to control that cross-links occur interchains (not intrachains), complete retention inside the polyplexes of unmodified siRNA is very challenging even for cross-linked systems.[18,56] Nevertheless, 40% of the total siRNA remained encapsulated in the decationized polyplexes.

In Vitro Cytotoxicity and Gene Silencing Activity of Decationized pHP-PEG-FA Polyplexes. pHP-PEG-FA decationized polyplexes were evaluated for cytotoxicity and gene silencing activity in the presence of serum using a folate receptor overexpressing cell line, that stably expresses firefly luciferase, Skov3-luc.

First, the potential in vitro toxicity of free pHP-PEG-FA polymer was analyzed by incubating the cells with solutions of a wide range of polymer concentrations (from 0.001 to 1 mg/

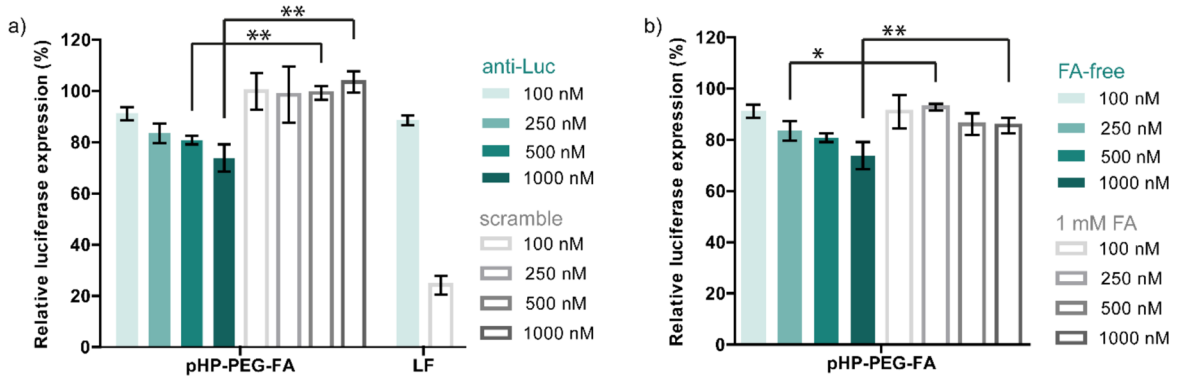


Figure 7. In vitro luciferase gene knockdown in Skov3-luc cells. (a) Cells were treated with decationized pHP-PEG-FA polyplex dispersions containing antiluciferase siRNA or scramble siRNA of different concentrations and (b) pHP-PEG-FA polyplexes containing antiluciferase siRNA in FA depleted medium and in FA saturated medium (1 mM). Results were normalized to buffer treated samples, and lipofectamine 2000 (LF) was used as a positive control for transfection (100 nM siRNA). Results are expressed as mean ± SD (n = 4). *p < 0.05; **p < 0.01 (t test, n = 4).

mL) (Figure S2 of the Supporting Information). Possible cytotoxic effects of free pHP-PEG-FA were evaluated by studying the interference on the intrinsic expression of luciferase by the cells (Figure S2a of the Supporting Information) and then by determining the interference on the cell metabolic activity determined by the XTT assay (Figure S2b of the Supporting Information). Results showed that that up to 1 mg/mL, neither interference on luciferase expression nor on the cell metabolic activity were detected, demonstrating that the free polymer possesses a very good cytocompatibility.

The cytotoxicity was also evaluated for siRNA pHP-PEG-FA decationized polyplexes with the XTT assay (Figure S3 of the Supporting Information). Again, cell viability was maintained close to 100%, even at high decationized polyplex concentrations (1000 nM siRNA, ~0.05 mg/mL pHP-PEG-FA). In line with previous findings, where high cytocompatibility was observed for pHP-PEG systems,[33,35] the results of the present study show that the safety profile was preserved with increasing the cross-link density of polyplexes.

Transfection efficiency of targeted decationized pHP-PEG-FA polyplexes was evaluated by their ability to induce luciferase gene silencing in Skov3-luc. Figure 7 shows that with increasing the anti-Luc siRNA polyplex dose, luciferase expression was reduced to 75% at the highest concentration (1000 nM siRNA). In contrast, cells treated with polyplexes loaded with scramble siRN, A luciferase expression was kept close to 100% luciferase, even at the highest SiRNA concentration of 1000 nM siRNA (Figure 7a). These results suggest that the reduction of luciferase expression observed in cells treated with siRNA was due to sequence-specific gene silencing.

The targeting ability of pHP-PEG-FA polyplexes was evaluated by anti-Luc siRNA polyplexes in the presence of FA saturated medium. Indeed, almost no silencing was observed even at the highest doses (Figure 7b) and demonstrates the potential of cell-specific transfection of folate targeted decationized polyplexes, as previously observed for pDNA polyplexes.[35] It should be noted that at the same siRNA dose, pHP-PEG-FA decationized polyplexes possessed a lower silencing efficiency than the lipofectamine siRNA formulation (Figure 7a). This high transfection activity of lipofectamine formulations has been previously found and is ascribed to their high degree of unspecific interaction with the transfected cells and also to their ability to later cluster in the cytoplasm.[14] It should also be noted that the transfection protocol using lipofectamine requires the absence of serum, which questions its applicability for in vivo studies and clinical translation. It is therefore remarkable that the transfection of pHP-PEG-FA polyplexes was evaluated in the presence of serum.

Folate-targeted nanoparticles bind with high affinity to the folate receptor of the cells and are internalized by receptor-mediated endocytosis. These folate-conjugates remain in "recycling" endosomes or eventually escape into the cytoplasm.[36,37,58] For efficient nucleic acid delivery (DNA or RNA based), polyplexes (including folate targeted systems[40]) which end up in endosomes require endosomal escape.[59−65] As demonstrated in previous studies, pHP-PEG-FA-based polyplexes do not possess intrinsic endosomal escape functionalities, which limits their efficiency at low concentrations.[33] Importantly, the high flexibility of this system allows the incorporation of more complex functionalities that can potentially improve the transfection efficiency of decationized polyplexes without compromising their safety profile. This can be achieved not only by improving endosmal escape of internalized polyplexes but also by improving the siRNA retention within the decationized polyplexes. For example, core−shell interface cross-linking can be exploited to increase the retention of loaded si-RNA molecules. Other possibilities include strategies directed to siRNA modification, for example, by multimerization of siRNA via disulfide linkages[54,66] or by preparation of polymer-siRNA[67] and endosomolytic peptide-siRNA conjugates (which can directly improve endosomal escape).[39] Increasing the hydrophobicity of the core of polyplexes, by introduction of hydrophobic groups, can further improve the stability of polyplexes. The hydrophobic monomer butyl methacrylate (BMA), for example, has the additional advantage to also give tuned pH-dependent membrane disruptive behavior and thus to trigger endosomal escape of polyplexes.[11]

■ CONCLUSIONS

In this study, folate targeted decationized polyplexes for siRNA delivery were developed. Polymer structure and polyplex preparation protocols were optimized to efficiently entrap the 21 bp structure of siRNA into nanosized polyplexes based on neutral polymers. The siRNA entrapment was mainly achieved by the presence of interchain disulfide cross-links in the core of the polyplexes, which provides stability in the extracellular environment and simultaneously allows an intracellular triggered release. As determined by FCS, even when incubated

in human plasma, polyplexes retained a substantial part of their siRNA loading. siRNA decationized polyplexes tested in vitro in a folate receptor overexpressing cell line stably expressing luciferase, Skvo3-luc, showed sequence specific gene silencing without detected cytotoxicity.

siRNA decationized polyplexes are ∼100−120 nm in diameter with a slightly negative zeta potential, which is the optimal feature to reduce off-target accumulation and retention upon systemic administration, particularly in the kidney. Future studies will be focused on investigation, whether the decationized polyplexes (and further optimized versions) can effectively accumulate and provide therapeutic effect at their target sites in in vivo models after systemic administration. Such studies can provide a deeper understanding of the therapeutic potential of these systems. The results of this study show that pHP-PEG-FA decationized polyplexes are a promising platform for siRNA delivery.

■ ASSOCIATED CONTENT

Supporting Information

Time-dependent siRNA released from decationized pHP-PEG-FA polyplexes; luciferase espression and relative metabolic activity determined by XTT of Skov3-luc cells upon treatment decationized pHP-PEG-FA polymer at different concentrations; and relative metabolic activity determined by XTT of Skov3-luc cells upon treatment decationized pHP-PEG-FA polyplexes at different concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

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

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

L. Novo is financially supported by Fundação para a Ciência e a Tecnologia (FCT), the Portuguese Foundation for Science and Technology, Grant SFRH/BD/47085/2008.

■ ABBREVIATIONS

ABCPA, 4,4′-azobis(4-cyanovaleric acid); BMA, butyl methacrylate; CMV, human cytomegalovirus promoter; DMAE, dimethylaminoethanol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DODT, 3,6-dioxa-1,8-octane-dithiol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EtOH, ethanol; FA, folic acid; FBS, fetal bovine serum; FCS, fluorescence correlation spectroscopy; GPC, gel permeation chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPMA, *N*-(2-hydroxypropyl)-methacrylamide; HPMA-DMAE, 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester; M_n, number-averaged molecular weight; mRNA, mRNA; M_w, weight-average molecular weight; MWCO, molecular weight cutoff; NH_4Ac, ammonium acetate; NMR, nuclear magnetic resonance; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; PDI, polydispersity index; pDNA, plasmid DNA; PDS, pyridyl disulfide; PDTEMA, *N*-[2-(2-pyridyldithio)]ethyl methacrylamide; PEG, poly(ethylene glycol); PMS, dibenzopyrazine methylsulfate; RI, refractive index; RISC, RNA-induced silencing complex; RLU, relative light units; RPMI, Roswell Park Memorial Institute medium; SD, standard deviation; siRNA, small interfering RNA; TAE, tris-acetate-EDTA; TAPS, 3-[[1,3-dihydroxy-2-(hydroxymethyl)-propan-2-yl]amino]propane-1-sulfonic acid; TCEP, tris(2-carboxyethyl)phosphine; TCSPC, time-correlated single photon counting; TEM, transmission electron microscopy; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

■ REFERENCES

(1) Daka, A.; Peer, D. RNAi-based nanomedicines for targeted personalized therapy. *Adv. Drug Delivery Rev.* **2012**, *64* (13), 1508−1521.

(2) Videira, M.; Arranja, A.; Rafael, D.; Gaspar, R. Preclinical development of siRNA therapeutics: Towards the match between fundamental science and engineered systems. *Nanomedicine* **2014**, *10* (4), 689−702.

(3) Hannon, G. J. RNA interference. *Nature* **2002**, *418* (6894), 244−251.

(4) Whitehead, K. A.; Langer, R.; Anderson, D. G. Knocking down barriers: Advances in siRNA delivery. *Nat. Rev. Drug Discovery* **2009**, *8* (2), 129−138.

(5) Oh, Y.-K.; Park, T. G. siRNA delivery systems for cancer treatment. *Adv. Drug Delivery Rev.* **2009**, *61* (10), 850−862.

(6) Davis, M. E. The First targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: From concept to clinic. *Mol. Pharmaceutics* **2009**, *6* (3), 659−668.

(7) Sato, A.; Choi, S. W.; Hirai, M.; Yamayoshi, A.; Moriyama, R.; Yamano, T.; Takagi, M.; Kano, A.; Shimamoto, A.; Maruyama, A. Polymer brush-stabilized polyplex for a siRNA carrier with long circulatory half-life. *J. Controlled Release* **2007**, *122* (3), 209−216.

(8) Mao, S.; Neu, M.; Germershaus, O.; Merkel, O.; Sitterberg, J.; Bakowsky, U.; Kissel, T. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-graft-poly(ethylene glycol) block copolymer/siRNA polyplexes. *Bioconjugate Chem.* **2006**, *17* (5), 1209−1218.

(9) de Wolf, H. K.; Snel, C. J.; Verbaan, F. J.; Schiffelers, R. M.; Hennink, W. E.; Storm, G. Effect of cationic carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous administration. *Int. J. Pharm.* **2007**, *331* (2), 167−175.

(10) Zuckerman, J. E.; Choi, C. H. J.; Han, H.; Davis, M. E. Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109* (8), 3137−3142.

(11) Nelson, C. E.; Kintzing, J. R.; Hanna, A.; Shannon, J. M.; Gupta, M. K.; Duvall, C. L. Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo. *ACS Nano* **2013**, *7* (10), 8870−8880.

(12) Naeye, B.; Deschout, H.; Caveliers, V.; Descamps, B.; Braeckmans, K.; Vanhove, C.; Demeester, J.; Lahoutte, T.; De Smedt, S. C.; Raemdonck, K. In vivo disassembly of IV administered siRNA matrix nanoparticles at the renal filtration barrier. *Biomaterials* **2013**, *34* (9), 2350−2358.

(13) Merkel, O. M.; Librizzi, D.; Pfestroff, A.; Schurrat, T.; Béhé, M.; Kissel, T. In vivo SPECT and real-time gamma camera imaging of biodistribution and pharmacokinetics of siRNA delivery using an optimized radiolabeling and purification procedure. *Bioconjugate Chem.* **2008**, *20* (1), 174−182.

(14) Kwok, A.; Hart, S. L. Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. *Nanomedicine* **2011**, *7* (2), 210−219.

(15) Manickam, D. S.; Li, J.; Putt, D. A.; Zhou, Q.-H.; Wu, C.; Lash, L. H.; Oupický, D. Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *J. Controlled Release* **2010**, *141* (1), 77−84.

(16) Breunig, M.; Hozsa, C.; Lungwitz, U.; Watanabe, K.; Umeda, I.; Kato, H.; Goepferich, A. Mechanistic investigation of poly(ethylene

imine)-based siRNA delivery: Disulfide bonds boost intracellular release of the cargo. *J. Controlled Release* **2008**, *130* (1), 57−63.
(17) Matsumoto, S.; Christie, R. J.; Nishiyama, N.; Miyata, K.; Ishii, A.; Oba, M.; Koyama, H.; Yamasaki, Y.; Kataoka, K. Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules* **2008**, *10* (1), 119−127.
(18) Christie, R. J.; Matsumoto, Y.; Miyata, K.; Nomoto, T.; Fukushima, S.; Osada, K.; Halnaut, J.; Pittella, F.; Kim, H. J.; Nishiyama, N.; Kataoka, K. Targeted polymeric micelles for siRNA treatment of experimental cancer by intravenous injection. *ACS Nano* **2012**, *6* (6), 5174−5189.
(19) Meng, F.; Hennink, W. E.; Zhong, Z. Reduction-sensitive polymers and bioconjugates for biomedical applications. *Biomaterials* **2009**, *30* (12), 2180−2198.
(20) Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Controlled Release* **2006**, *114* (1), 100−109.
(21) Duncan, R. The dawning era of polymer therapeutics. *Nat. Rev. Drug Discovery* **2003**, *2* (5), 347−360.
(22) Jones, C. F.; Campbell, R. A.; Brooks, A. E.; Assemi, S.; Tadjiki, S.; Thiagarajan, G.; Mulcock, C.; Weyrich, A. S.; Brooks, B. D.; Ghandehari, H.; Grainger, D. W. Cationic PAMAM dendrimers aggressively initiate blood clot formation. *ACS Nano* **2012**, *6* (11), 9900−9910.
(23) Luten, J.; van Nostrum, C. F.; De Smedt, S. C.; Hennink, W. E. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. *J. Controlled Release* **2008**, *126* (2), 97−110.
(24) van der Aa, L. J.; Vader, P.; Storm, G.; Schiffelers, R. M.; Engbersen, J. F. J. Optimization of poly(amido amine)s as vectors for siRNA delivery. *J. Controlled Release* **2011**, *150* (2), 177−186.
(25) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, *24* (7), 1121−1131.
(26) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. A two-stage poly(ethylenimine)-mediated cytotoxicity: Implications for gene transfer/therapy. *Mol. Ther.* **2005**, *11* (6), 990−995.
(27) Choksakulnimitr, S.; Masuda, S.; Tokuda, H.; Takakura, Y.; Hashida, M. In vitro cytotoxicity of macromolecules in different cell culture systems. *J. Controlled Release* **1995**, *34* (3), 233−241.
(28) Lonez, C.; Vandenbranden, M.; Ruysschaert, J.-M. Cationic lipids activate intracellular signaling pathways. *Adv. Drug Delivery Rev.* **2012**, *64* (15), 1749−1758.
(29) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. *Biomaterials* **2001**, *22* (5), 471−480.
(30) Masago, K.; Itaka, K.; Nishiyama, N.; Chung, U.-i.; Kataoka, K. Gene delivery with biocompatible cationic polymer: Pharmacogenomic analysis on cell bioactivity. *Biomaterials* **2007**, *28* (34), 5169−5175.
(31) Merkel, O. M.; Beyerle, A.; Beckmann, B. M.; Zheng, M.; Hartmann, R. K.; Stöger, T.; Kissel, T. H. Polymer-related off-target effects in non-viral siRNA delivery. *Biomaterials* **2011**, *32* (9), 2388−2398.
(32) Regnström, K.; Ragnarsson, E. G. E.; Köping-Höggård, M.; Torstensson, E.; Nyblom, H.; Artursson, P. PEI, a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice. *Gene Ther.* **2003**, *10* (18), 1575−1583.
(33) Novo, L.; van Gaal, E. V. B.; Mastrobattista, E.; van Nostrum, C. F.; Hennink, W. E. Decationized crosslinked polyplexes for redox-triggered gene delivery. *J. Controlled Release* **2013**, *169* (3), 246−256.
(34) Novo, L.; Rizzo, L. Y.; Golombek, S. K.; Dakwar, G. R.; Lou, B.; Jahnen-Dechent, W.; Remaut, K.; Mastrobattista, E.; van Nostrum, C. F.; Kiessling, F.; Braeckmans, K.; Lammers, T.; Hennink, W. E. Decationized polyplexes as stable and safe carrier systems for improved biodistribution in systemic gene therapy. *J. Controlled Release* **2014**, DOI: 10.1016/j.jconrel.2014.08.028.
(35) Novo, L.; Mastrobattista, E.; van Nostrum, C. F.; Hennink, W. E. Targeted decationized polyplexes for cell specific gene delivery. *Bioconjugate Chem.* **2014**, *25* (4), 802−812.
(36) Hilgenbrink, A. R.; Low, P. S. Folate receptor-mediated drug targeting: From therapeutics to diagnostics. *J. Pharm. Sci.* **2005**, *94* (10), 2135−2146.
(37) Lu, Y.; Low, P. S. Folate-mediated delivery of macromolecular anticancer therapeutic agents. *Adv. Drug Delivery Rev.* **2012**, *64* (Supplement 0), 342−352.
(38) Benoit, D. S. W.; Srinivasan, S.; Shubin, A. D.; Stayton, P. S. Synthesis of folate-functionalized RAFT polymers for targeted siRNA delivery. *Biomacromolecules* **2011**, *12* (7), 2708−2714.
(39) Dohmen, C.; Edinger, D.; Fröhlich, T.; Schreiner, L.; Lächelt, U.; Troiber, C.; Rädler, J.; Hadwiger, P.; Vornlocher, H.-P.; Wagner, E. Nanosized multifunctional polyplexes for receptor-mediated siRNA delivery. *ACS Nano* **2012**, *6* (6), 5198−5208.
(40) Dohmen, C.; Frohlich, T.; Lachelt, U.; Rohl, I.; Vornlocher, H.-P.; Hadwiger, P.; Wagner, E. Defined folate-PEG-siRNA conjugates for receptor-specific gene silencing. *Mol. Ther. Nucleic Acids* **2012**, *1*, e7.
(41) Scholz, C.; Wagner, E. Therapeutic plasmid DNA versus siRNA delivery: Common and different tasks for synthetic carriers. *J. Controlled Release* **2012**, *161* (2), 554−565.
(42) Grassetti, D. R.; Murray, J. F. Determination of sulfhydryl groups with 2,2′- or 4,4′-dithiodipyridine. *Arch. Biochem. Biophys.* **1967**, *119*, 41−49.
(43) Ryu, J.-H.; Chacko, R. T.; Jiwpanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. Self-cross-linked polymer nanogels: A versatile nanoscopic drug delivery platform. *J. Am. Chem. Soc.* **2010**, *132* (48), 17227−17235.
(44) Buyens, K.; Lucas, B.; Raemdonck, K.; Braeckmans, K.; Vercammen, J.; Hendrix, J.; Engelborghs, Y.; De Smedt, S. C.; Sanders, N. N. A fast and sensitive method for measuring the integrity of siRNA-carrier complexes in full human serum. *J. Controlled Release* **2008**, *126* (1), 67−76.
(45) Funhoff, A.; van Nostrum, C. F.; Janssen, A.; Fens, M.; Crommelin, D.; Hennink, W. E. Polymer side-chain degradation as a tool to control the destabilization of polyplexes. *Pharm. Res.* **2004**, *21* (1), 170−176.
(46) Verbaan, F. J.; Oussoren, C.; Snel, C. J.; Crommelin, D. J. A.; Hennink, W. E.; Storm, G. Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. *J. Gene Med.* **2004**, *6* (1), 64−75.
(47) Ogris, M.; Brunner, S.; Schuller, S.; Kircheis, R.; Wagner, E. PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **1999**, *6* (4), 595−605.
(48) Harada-Shiba, M.; Yamauchi, K.; Harada, A.; Takamisawa, I.; Shimokado, K.; Kataoka, K. Polyion complex micelles as vectors in gene therapy: pharmacokinetics and in vivo gene transfer. *Gene Ther.* **2002**, *9* (6), 407−14.
(49) Walkey, C. D.; Chan, W. C. W. Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem. Soc. Rev.* **2012**, *41* (7), 2780−2799.
(50) Dakwar, G. R.; Zagato, E.; Delanghe, J.; Hobel, S.; Aigner, A.; Denys, H.; Braeckmans, K.; Ceelen, W.; De Smedt, S. C.; Remaut, K. Colloidal stability of nano-sized particles in the peritoneal fluid: Towards optimizing drug delivery systems for intraperitoneal therapy. *Acta Biomater.* **2014**, *10* (7), 2965−2975.
(51) Storm, G.; Belliot, S. O.; Daemen, T.; Lasic, D. D. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv. Drug Delivery Rev.* **1995**, *17* (1), 31−48.
(52) Lee, M. H.; Yang, Z.; Lim, C. W.; Lee, Y. H.; Dongbang, S.; Kang, C.; Kim, J. S. Disulfide-cleavage-triggered chemosensors and their biological applications. *Chem. Rev.* **2013**, *113* (7), 5071−5109.
(53) Wagner, E. Polymers for siRNA delivery: Inspired by viruses to be targeted, dynamic, and precise. *Acc. Chem. Res.* **2011**, *45* (7), 1005−1013.

(54) Lee, S.-Y.; Huh, M. S.; Lee, S.; Lee, S. J.; Chung, H.; Park, J. H.; Oh, Y.-K.; Choi, K.; Kim, K.; Kwon, I. C. Stability and cellular uptake of polymerized siRNA (poly-siRNA)/polyethylenimine (PEI) complexes for efficient gene silencing. *J. Controlled Release* **2010**, *141* (3), 339−346.
(55) Filipe, V.; Hawe, A.; Jiskoot, W. Critical evaluation of nanoparticle tracking analysis (NTA) by Nanosight for the measurement of nanoparticles and protein aggregates. *Pharm. Res.* **2010**, *27* (5), 796−810.
(56) Troiber, C.; Kasper, J. C.; Milani, S.; Scheible, M.; Martin, I.; Schaubhut, F.; Küchler, S.; Rädler, J.; Simmel, F. C.; Friess, W.; Wagner, E. Comparison of four different particle sizing methods for siRNA polyplex characterization. *Eur. J. Pharm. Biopharm.* **2013**, *84* (2), 255−264.
(57) Christie, R. J.; Miyata, K.; Matsumoto, Y.; Nomoto, T.; Menasco, D.; Lai, T. C.; Pennisi, M.; Osada, K.; Fukushima, S.; Nishiyama, N.; Yamasaki, Y.; Kataoka, K. Effect of polymer structure on micelles formed between siRNA and cationic block copolymer comprising thiols and amidines. *Biomacromolecules* **2011**, *12* (9), 3174−3185.
(58) Zhao, X.; Li, H.; Lee, R. J. Targeted drug delivery via folate receptors. *Expert Opin. Drug Delivery* **2008**, *5* (3), 309−319.
(59) Mintzer, M. A.; Simanek, E. E. Nonviral vectors for gene delivery. *Chem. Rev.* **2009**, *109* (2), 259−302.
(60) 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.; Crommelin, D. J. A. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. *Pharm. Res.* **2007**, *24* (8), 1590−1598.
(61) Yang, Z.; Sahay, G.; Sriadibhatla, S.; Kabanov, A. V. Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. *Bioconjugate Chem.* **2008**, *19* (10), 1987−1994.
(62) Vercauteren, D.; Rejman, J.; Martens, T. F.; Demeester, J.; De Smedt, S. C.; Braeckmans, K. On the cellular processing of non-viral nanomedicines for nucleic acid delivery: Mechanisms and methods. *J. Controlled Release* **2012**, *161* (2), 566−581.
(63) Meyer, M.; Philipp, A.; Oskuee, R.; Schmidt, C.; Wagner, E. Breathing life into polycations: Functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *J. Am. Chem. Soc.* **2008**, *130* (11), 3272−3273.
(64) Meyer, M.; Dohmen, C.; Philipp, A.; Kiener, D.; Maiwald, G.; Scheu, C.; Ogris, M.; Wagner, E. Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA−polymer conjugate. *Mol. Pharmaceutics* **2009**, *6* (3), 752−762.
(65) Yu, S. S.; Lau, C. M.; Barham, W. J.; Onishko, H. M.; Nelson, C. E.; Li, H.; Smith, C. A.; Yull, F. E.; Duvall, C. L.; Giorgio, T. D. Macrophage-specific RNA interference targeting via "Click", mannosylated polymeric micelles. *Mol. Pharmaceutics* **2013**, *10* (3), 975−987.
(66) Mok, H.; Lee, S. H.; Park, J. W.; Park, T. G. Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing. *Nat. Mater.* **2010**, *9* (3), 272−278.
(67) Takemoto, H.; Ishii, A.; Miyata, K.; Nakanishi, M.; Oba, M.; Ishii, T.; Yamasaki, Y.; Nishiyama, N.; Kataoka, K. Polyion complex stability and gene silencing efficiency with a siRNA-grafted polymer delivery system. *Biomaterials* **2010**, *31* (31), 8097−8105.