Influence of Polyethylene Glycol Chain Length on the Physicochemical and Biological Properties of Poly(ethylene imine)-*graft*-Poly(ethylene glycol) Block Copolymer/SiRNA Polyplexes

Shirui Mao,[†,‡] Michael Neu,[†] Oliver Germershaus,[†] Olivia Merkel,[†] Johannes Sitterberg,[†] Udo Bakowsky,[†] and Thomas Kissel*[,†]

Department of Pharmaceutics and Biopharmacy, Philipps Universität Marburg, Ketzerbach 63, 35032 Marburg, Germany, and School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, China. Received May 19, 2006; Revised Manuscript Received August 7, 2006

Polyplexes between siRNA and poly(ethylene imine) (PEI) derivatives are promising nonviral carriers for siRNA. The polyplex stability is of critical importance for efficient siRNA delivery to the cytoplasm. Here, we investigate the effect of PEGylation at a constant ratio (∼50%) on the biophysical properties of the polyplexes. Particle size, ζ potential, and stability against heparin as well as RNase digestion and reporter gene knockdown under in vitro conditions of different siRNA polyplexes were characterized. Stability and size of siRNA polyplexes were clearly influenced by PEI−PEG structure, and high degrees of substitution such as PEI(25k)-*g*-PEG(550)$_{30}$ resulted in large (300−400 nm), diffuse complexes (AFM) which showed condensation behavior only at high N/P ratios. All other polyplexes and the PEI control showed similar sizes (150 nm) and compact structures in AFM, with complete condensation reached at N/P ratio of 3. Stability of siRNA polyplexes against heparin displacement and RNase digestion could be modified by PEGylation. Protection against RNase digestion was highest for PEI(25k)-*g*-PEG(5k)$_4$ and PEI(25k)-*g*-PEG(20k)$_1$, while siRNA/PEI provided insufficient protection. In knockdown experiments using NIH/3T3 fibroblasts stably expressing *β*-galactosidase, it was shown that PEG chain length had a significant influence on biological activity of siRNA. Polyplexes with siRNA containing PEI(25k)-*g*-PEG(5k)$_4$ and PEI-(25k)-*g*-PEG(20k)$_1$ yielded similar efficiencies of ca. 70% knockdown as lipofectamine controls. Confocal microscopy demonstrated enhanced cellular uptake of siRNA into cytosol by polyplexes formation with PEI copolymers. In conclusion, both the chain length and graft density of PEG were found to strongly influence siRNA condensation and stability and hence affect the knockdown efficiency of PEI−PEG/siRNA polyplexes.

INTRODUCTION

RNA interference (RNAi) using small interfering RNA (siRNA, a double-stranded RNA with 21−23 bp) has recently emerged as a powerful tool for silencing target genes and holds great possibility for therapeutic application (*1*). The first clinical data on RNAi have been published recently (*2*). However, to achieve efficient gene knockdown under in vivo conditions, siRNA degradation in the physiological milieu, poor cellular uptake, and inefficient translocation into the cytoplasm need to be improved. Macromolecules like siRNA are typically internalized by endocytosis followed by lysosomal fusion causing siRNA degradation. Endosomal escape is a prerequisite for efficient delivery of intact siRNA to the cytosol, its site of biological action (*3*). Moreover, the polyanionic nature of siRNA impairs diffusion through cell membranes and cellular uptake. Therefore, delivery systems for siRNA are of particular importance to exploit their therapeutic potential (*4*). A promising approach could be siRNA delivery through complexation of siRNA using biodegradable and biocompatible polycations (*5*).

Poly(ethylene imine) (PEI) is well-known as an efficient nonviral nucleic acid vector demonstrating high transfection rates under in vitro and in vivo conditions (*6*). PEI forms polyplexes with anionic nucleotides by electrostatic interactions. The strong buffer capacity, designated as the proton sponge hypothesis, could be responsible for the fact that PEI-based delivery systems do not require endosome disruptive agents for lysosomal escape (*7*). Once released from endosomes, the siRNA may enter the cytosol, where it associates with its complementary siRNA strand and, depending on its complementarity, results in either targeting cleavage of the mRNA or translational arrest. However, intact PEI/plasmid complexes were found in the cell nucleus (*8*, *9*), implying that PEI/siRNA polyplexes might be too stable to be released in the cytosol and will be transported into the nucleus, as in the case of plasmid/PEI complexes (*8*); therefore, no knockdown will be observed. To guarantee the biological activity of siRNA, this point needs to be clarified, and stability of the PEI/siRNA polyplexes needs to be further modified. The polyplexes should be sufficiently stable during the endocytosis process to ensure sufficient intracellular uptake of siRNA, but thereafter, siRNA should be released into the cytosol. While it was noticed that a significant decrease in transfection activity for the PEI−PEG/DNA polyplexes was found (*10*, *11*), it is reasonable to assume that PEGylation might be able to destabilize PEI/siRNA polyplexes, leading to increased siRNA release in the cytoplasm and therefore efficient knockdown. This hypothesis was investigated in this study.

Recently, application of linear PEI or low molecular PEI as an siRNA delivery vector has been reported (*12*−*15*). In agreement with our hypothesis, linear PEI was efficient for delivering nucleic acids to cells but did not lead to siRNA activity within the applied dose of <0.5 pmol (*12*). Direct intratumor injection of a siRNA-encoding plasmid complexed

* To whom correspondence should be addressed. Department of Pharmaceutics and Biopharmacy, Phillips-Universität Marburg, Ketzerbach 63, D-35032 Marburg, Germany, Tel +49-6421-282-5881, FAX +49-6421-282-7016, E-mail: kissel@staff.uni-marburg.de.

[†] Philipps Universität Marburg.

[‡] Shenyang Pharmaceutical University.

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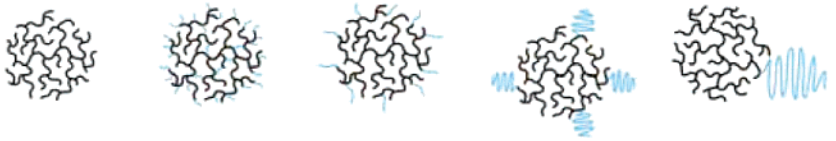
Table 1. Schematic Structure and Properties of the PEI-*g*-PEG Copolymers Investigated in This Study[a]

Compound	PEI	PEI (25k)-g -PEG(550)$_{30}$	PEI(25k)-g -PEG(2k)$_{10}$	PEI(25k)-g -PEG(5k)$_4$	PEI(25k)-g -PEG(20k)$_1$
PEI content	100%	60%	55%	55%	55%
Mw PEI	25000	25000	25000	25000	25000
Mw PEG		550	2000	5000	20000
Mw	25000	41500	45000	45000	45000
Structure					

[a] The black part of the structure represents the branched PEI 25 kDa, and the blue part represents the nonionic linear PEG segments.

with linear PEI efficiently reduced the vascularization of treated tumors but was not able to reduce tumor growth (*13*). The low activity of siRNA might be attributed to insufficient release in the cytosol.

PEGylated PEI was synthesized in our group to decrease the cytotoxicity of branched PEI and was extensively studied as vectors for DNA delivery (*16*). Moreover, strong evidence was provided that PEGylated PEI could be adapted to function as an effective carrier for cellular delivery of small oligonucleotides. Recent reports have characterized the properties and transfection capacity of PEGylated PEI25k complexed with oligonucleotides (*17*, *18*). Similarly, branched PEI-*graft*-poly(ethylene glycol) has been recently reported as a carrier of siRNA with different targeting ligands, and the results are promising (*19*−*21*).

Regarding PEGylation, both PEG chain length and graft density clearly affect pharmacokinetic properties of polyplexes containing nucleic acids, although in vivo results are discussed controversially. While some authors suggest that low graft densities with high molecular weight PEG are more effective in reducing protein adsorption than the higher surface density of low molecular weight polymers (*22*), others found that density of PEG grafting on the surface of polyplexes had a more pronounced effect on protein adsorption than PEG chain length on steric repulsion and van der Waals attraction (*23*). For DNA polyplexes, a sufficiently high graft density with PEG molecules seems to be necessary to prevent opsonization and avoid rapid clearance from the blood stream (*17*). However, little information is available regarding the influence of PEG chain length on the physicochemical and biological properties of nucleic nanoparticles (*17*).

Therefore, the objective of this study was to evaluate the stability of PEI and PEI−PEG polyplexes with siRNA using biophysical methods and to elucidate the influence of PEG chain length and graft density. Our hypothesis was tested with a panel of PEI−PEGs having similar composition (50% PEG content) but vastly different structures. Here, PEI(25k)-*g*-PEG copolymers with PEGylation degree in the range 40−60% and different PEG chain lengths (550, 2k, 5k, 20k, respectively) were selected and compared with that of the homopolymer PEI(25k). This selection is based on the fact that the molecular weights of linear PEGs used in biomedical applications usually range between a few hundred to 20 000 Da. The relationship between polymer structure and stability of the polyplexes was investigated. Furthermore, the knockdown efficiency of the selected polyplexes was studied in cell culture to find the correlation between in vitro stability and biological effect. Uptake and distribution of the polyplexes in cells were visualized by confocal laser scanning microscopy. Due to the different properties of siRNA compared to DNA (*24*), this study should provide a basis for the design of siRNA delivery systems.

MATERIALS AND METHODS

Materials. Branched poly(ethylene imine), PEI, with a molecular weight of 25 kDa was purchased from Sigma-Aldrich (Seelze, Germany). The poly(ethylene imine)-*graft*-poly(ethylene glycol) (PEI−PEG) with a PEG content of approximately 50% (w/w) was synthesized as previously described (*16*) by grafting linear PEG of 550 Da and 2, 5, and 20 kDa, respectively, onto branched PEI 25 kDa. Polymers were stored as sterile filtered, aqueous stock solutions (5 mg/mL) at pH 7.4. These graft copolymers were designated using the following nomenclature: PEI(25k)-*g*-PEG(x)$_n$. The number in brackets (25k or x, where x = 550, 2k, 5k, or 20k) represents the MW of PEI or PEG block in daltons, and the index n is the average number of PEG blocks per PEI molecule. This number was calculated on the basis of ^{1}H NMR spectra as described previously (*16*). The schematic structure and properties of the copolymers investigated in this paper are shown in Table 1. siGL3 (luciferase GL3 Duplex) was purchased from Dharmacon (Lafayette, CO) and used for physicochemical studies. The sequence is as follows: 5′-CUU ACG CUG AGU ACU UCG A dTdT/ dTdT GAA UGC GAC UCA UGA AGC U-5′. For the knockdown experiment in cell culture, an anti β-Gal siRNA duplex was purchased from Dharmacon with the following sequence: 5′-CUA CAC AAA UCA GCG AUU U UU /UU GAU GUG UUU AGU CGC UAA A P-5′. Cyanine 3 (Cy3)-labeled siRNA was from Dharmacon (Lafayette, CO). RNase A for the RNase digestion study was purchased from Roche Diagnostics GmbH, Mannheim, Germany. All other materials were obtained in analytical quality.

Preparation and Characterization of Polymer/siRNA Polyplexes. All polyplexes consisting of siRNA and PEI derivatives were prepared in isotonic glucose solution at pH 7 unless otherwise mentioned. The polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio, where N represents moles of amine on PEI and P represents moles of phosphate on siRNA. On the basis of the N/P ratio, the appropriate amount of polymer was added to the siRNA solution, mixed by vigorous pipetting, and incubated for 10 min at room temperature.

The hydrodynamic diameter of freshly prepared polyplexes was measured using a Zetasizer Nano ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm. Measurements were

performed at 25 °C and analyzed in CONTIN mode. The instrument was checked periodically with nanospheres size standards (polymer microspheres in water, 199 nm ± 6 nm) from Duke Scientific (Palo Alto, CA). For data analysis, the viscosity and refractive index of water at 25 °C (0.89 mPa·s and 1.333, respectively) were used. The measurements were performed in triplicate. The ζ potentials (surface charge) of freshly prepared polyplexes were determined using the standard capillary electrophoresis cell of Zetasizer Nano ZS from Malvern Instrument at 25 °C. Measurements were performed in glucose 5%, pH 7.4. Average values were calculated with the data of 10 runs with standard deviation.

Atomic Force Microscopy (AFM). The polyplexes were prepared as described above and diluted in milliQ water. AFM images were obtained on a NanoWizard (JPK instruments, Berlin, Germany). The microscope was vibration-damped. Commercial pyramidal tips (Micromash, Estonia) on an I-tape cantilever with a length of about 230 μm, a resonance frequency of about 160 kHz, and a nominal force constant of 40 N/m were used. All measurements were performed in intermittent-contact mode at a scan speed of approximately 1 Hz to avoid damage of the sample surface. The acquired pictures had a resolution of 512 × 512 pixels. Polyplexes were directly transferred onto a prewashed silicon chip by dipping the chip into the polyplex solution for 10 min, rinsing it with MilliQ water, and allowing it to dry in air.

SiRNA Condensation. To study siRNA condensation as a function of N/P ratio, polyacrylamide gel (20%) electrophoresis (PAGE) retardation assays were performed (*18*). Briefly, polyplexes preparation was carried out in 5% glucose solution at pH 7.0. Increasing amounts of PEI were added to 6 pmol siRNA to reach final N/P ratios of 0.5, 0.7, 1, 1.5, 2, 3, and 5 for PEI 25k and 2, 3, 5, 7, 10, 15, and 20 for other copolymers. The resulting polyplexes were incubated for 10 min prior to loading to the gel. Then, the gel electrophoresis was run in TBE buffer pH 8 at a constant current of 15 mA. To visualize siRNA, the gel was immersed in 0.0017% ethidium bromide solution for 5 min. Thereafter, fluorescence of intercalated ethidium bromide was detected using a transilluminator (BioDoc Analyze, Biometra, Goettingen, Germany).

Polyanion Competition Assay. The relative stability of polyplexes was tested by measuring siRNA release from polyplexes in the presence of a competing polyanion heparin (*25*). All polyplexes were formed at N/P of 10. Polyplex solutions were incubated in the presence of 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, and 0.15 IU of heparin/μg siRNA, respectively. These solutions were mixed well and incubated for 10 min before being applied to a 20% PAGE. In each gel, free siRNA was applied as a reference. Gels were run for 2 h at 15 mA and then scanned using a Biometra gel analyzing system. The experiments were performed in triplicate.

RNase Digestion. Stability of siRNA polyplexes against RNase digestion was investigated according to a method described previously (*26*) with little modification. The protection effect of different siRNA/PEI derivative polyplexes against series RNase digestion was investigated. Briefly, polyplexes were prepared at N/P of 10 in glucose 5%. Aliquots of 12.5 μL polyplexes corresponding to 0.17 μg of siRNA were incubated with 0.05, 0.10, 0.20, 0.50, 0.70, 1.0, and 1.2 mIU of RNase in phosphate buffer at pH 7.0 for 30 min at 37 °C. Afterward, samples were incubated for a further 30 min at 70 °C to inactivate RNase and divided into two equal fractions. The first fraction was used for determination of integrity of the polyplexes and the second fraction with 2 μL of a heparin solution containing 1000 IU per mL to release of the amount of siRNA remaining intact. Resulting mixtures were applied to a 20% PAGE gel, and electrophoresis was carried out at 15 mA for 2 h. A positive control containing free siRNA was tested under the same conditions. The resulting gel was imaged on a Biometra transilluminator. The intensity of the "free siRNA" band was quantified by densitometry using the software program *Scion Image 4.0.2. beta* (Scion Corporation, Frederick, MD). At a given RNase concentration, the percentage of siRNA dissociated from the polyplexes was obtained by normalizing against the siRNA band measured from a sample containing siRNA only on the same gel. All experiments were performed in triplicate.

Knockdown Experiments in NIH 3T3 Fibroblasts under in Vitro Conditions. The knockdown experiment was performed as described in the literature (*27*). Briefly, 24 h before transfection, NIH/3T3 cells stably expressing β-galactosidase (Orbigen, San Diego, CA) were seeded in 24-well plates at a density of 3×10^4 per well in 500 μL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 (v/v). Cells were at 50−80% confluence before experiment. siRNA polyplexes with different polymers were prepared at N/P of 10 and incubated for 10 min at room temperature. The total volume of the polyplexes was 100 μL, and the polyplexes were added to the cells in 400 μL DMEM/10% FBS (50 pmol siRNA per well). For lipofectamine 2000, the method was similar except that the lipofectamine 2000/siRNA ratio was 2:1. After 4 h incubation, the medium was changed, and cells were further incubated for 20 h. Thereafter, the cells were washed with PBS and then were lysed with 100 μL of lysis buffer (Galacto-Star System, Applied Biosystems, Bedford, MA). Protein concentration was measured by using the BCA assay kit. β-Galactosidase activity was measured with a luminometer. Relative light units were converted to picograms of β-galactosidase by using β-galactosidase as a standard. The experiments were performed in triplicate.

Preparation of FITC-Labeled Copolymer. Copolymer PEI(25k)-*g*-PEG(5k)$_4$ was labeled with fluorescein isothiocyanate (FITC) for cellular trafficking studies. Briefly, 10 mg PEI(25k)-*g*-PEG(5k)$_4$ was dissolved in 2 mL of 0.1 M sodium hydrogen carbonate (pH 8.2). FITC (1 mg in 200 μL water-free DMSO) was added to the solution dropwise under stirring, and the mixture was stirred at room temperature for 4 h under light exclusion. Purification was performed on a PD-10 column (Amersham Pharmacia Biotech, Freiburg, Germany).

Confocal Laser Scanning Microscopy (CLSM). Confocal laser scanning microscopy was utilized to visualize the internalization of siRNA polyplexes. NIH/3T3 cells stably expressing β-galactosidase were seeded at a density of 12 000 cells per well in 8-well chamber slides (LabTek, Nunc, Wiesbaden, Germany). After 24 h, medium was removed and polyplexes containing FITC-labeled PEI(25k)-*g*-PEG(5k)$_4$, and Cyanine 3 labeled siRNA, free Cyanine 3 labeled siRNA, and free FITC-labeled polymer were added in fresh media. After 2 h incubation, cells were washed three times with phosphate buffered saline (PBS). Fixation of cells was done by incubation with 4% paraformaldehyde solution in PBS pH 7.4 at room temperature for 30 min and counterstaining with DAPI (4,6-diamidino-2-phenylindole, 0.2 μg/mL) for 20 min, both under light exclusion. Cells were embedded in FluorSave Reagent (Calbiochem, San Diego, CA). A Zeiss Axiovert 100 M microscope coupled to a Zeiss LSM 510 scanning device (Zeiss, Oberkochen, Germany) was used for confocal microscopy. For excitation of Cy3 fluorescence, a helium−neon laser with an excitation wavelength of 543 nm was used. Fluorescence emission was detected using a 560−615 nm band-pass filter. In a second scan, FITC was detected using an argon laser with an excitation wavelength of 488 nm and a 505−550 nm band-pass emission filter. Simultaneously, transmitted light images were obtained in this scan. In a third scan, DAPI was excited at 364 nm using an Enterprise

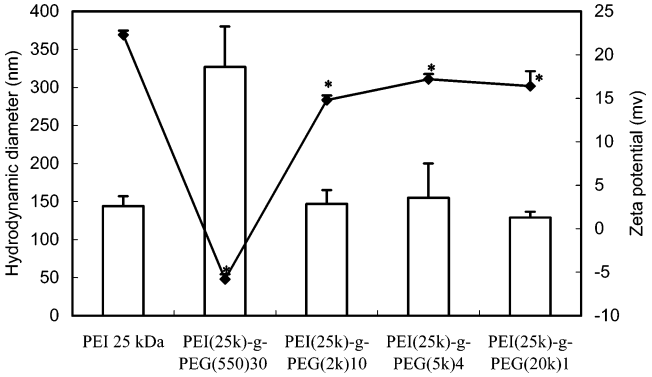


Figure 1. Physicochemical properties of PEI−PEG/siRNA (siGL3) polyplexes in 5% glucose at N/P ratio of 10. (◆) ζ potential; (bar) particle size. *$P < 0.05$.

II 653 laser (Coherent Inc., Santa Clara, CA), and fluorescence was detected using a 385−470 nm band-pass filter.

Statistics. The results are reported as mean ± standard deviation (SD) of at least three experiments. Significance between the mean values was calculated using one-way ANOVA analysis (*Origin 7.0 SRO*, Northampton, MA). Probability values $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

With PEG weight ratio (~50%) kept constant, branched PEI 25 kDa was grafted with PEG 0.55k, 2k, 5k, and 20k, respectively, and the influence of PEG chain length on the stability and biological activity of the resulting siRNA polyplexes was studied. We hypothesized that PEGylation might modify the stability of the polyplexes and therefore affect the knockdown efficiency of siRNA by increasing the proportion of siRNA released in the cytoplasm avoiding uptake into the nucleus.

Characterization of the Polyplexes. The hydrodynamic diameters of PEI−PEG/siGL3 polyplexes were determined at nitrogen-to-phosphate (N/P) ratio of 10. In preliminary experiments with the copolymer PEI(25k)-*g*-PEG$(2k)_{10}$, it was shown that polyplex sizes decreased with increasing N/P ratios from 5 to 10 and remained constant up to N/P of 50. Therefore, N/P of 10 was adopted for size measurements. Properties of PEI−PEG/siGL3 polyplexes in 5% glucose are shown in Figure 1. The influence of PEG chain length could be seen in the way that the copolymer with fewer PEG blocks formed smaller polyplexes than copolymers with more PEG blocks. The homopolymer PEI exhibited a particle size of 144 nm. In contrast, copolymer PEI(25k)-*g*-PEG$(550)_{30}$ formed large polyplexes of 300−400 nm even at N/P ratio of 20, probably due to a decreased condensation efficiency of this highly grafted derivative, as indicated in the siRNA retardation assay. The copolymers with PEG chains 2k and 5k formed polyplexes of appropriately 150 nm, and slightly decreased particles size was found with copolymer PEI(25k)-*g*-PEG$(20k)_1$, with particles size around 130 nm.

The enhanced siRNA complexation might be attributed to the special copolymer structure. With only one PEG block on average, this is a diblock-like copolymer, and there might be a PEI domain and a PEG domain clearly separated from each other. Thus, the siRNA seems to interact with the PEI domain without interference from PEG. However, no statistical difference was found among the particle sizes of polyplexes prepared with copolymers of PEG chains 2k, 5k, and 20k. A similar trend was reported for NF-kB decoy and DNA PEI−PEG complexes (*17*, *28*). Moreover, the stability of the polyplexes was investigated for 20 min. It is very important to note that all the siRNA/copolymer polyplexes remained stable after 20 min incubation (data not shown). In comparison, the size of PEI(25k)/siRNA polyplexes increased approximately 35%. This can be explained by the steric shielding effect of PEG, thereby abolishing aggregation. This effect has been demonstrated for PEGylated PEIs in other studies (*28*). In addition, PEG might additionally participate in the siRNA condensation process in a synergistic manner (*29*). In contrast, particle size of PEI-*g*-PEG/oligonucleotides was reported to be less than 100 nm in 150 mM sodium chloride (*18*), and this was attributed to differing sequences of the nucleic acid and different media used in this study. To clarify this point, hydrodynamic diameters of PEI−PEG/siGL3 polyplexes were also investigated in 150 mM sodium chloride solution. Aggregation appeared immediately after preparation of polyplexes with particle sizes between 500 and 1000 nm. These data confirm that properties of the polyplexes were siRNA sequence- and medium-dependent. Similarly, particle size of β-Gal siRNA polyplexes of 400−900 nm was reported recently, which was measured in 20 mM HEPES buffer after incubating in OptiMEM medium for 30 min (*30*).

Interactions between polymer/siRNA polyplexes and cell surfaces are dependent upon the charge of the particles. The surface charges of PEI−PEG/siRNA polyplexes are described in Figure 1. PEI 25 kDa polyplexes revealed high positive surface charge of +22.3 mV. All copolymers showed a significantly reduced ζ potential compared to PEI. Full shielding was observed with copolymer PEI(25k)-*g*-PEG$(550)_{30}$. No statistically significant difference was found for the copolymers with PEG$(2k)_{10}$, PEG$(5k)_4$, and PEG$(20k)_1$, implying that at least part of the PEG blocks of the copolymers orientate toward the surface while PEI and siRNA are located in the inner part of the polyplexes. Therefore, it is reasonable to conclude that the PEG protective effect is caused by a combination of PEG chain length and density; a threshold PEG length is needed for higher efficiency.

Atomic Force Microscopy. Atomic force microscopy (AFM) was used to study the morphology of polymer/siRNA polyplexes and compared with that of free siRNA, as shown in Figure 2. Polyplexes were imaged at an N/P ratio of 10 except PEI−PEG(550) (N/P 20). As anticipated, a double-stranded structure was found for free siRNA duplex (Figure 2a). After complexation with the polymers, the double-stranded structure disappeared, and all the polyplexes were spherical or subspherical with smooth surfaces (Figure 2). The homopolymer PEI 25 kDa showed images of compact homogeneously distributed polyplexes, with some large particles. PEGylation affected the properties of the polyplexes considerably. Particle aggregation was quite serious in the case of PEI−PEG(550) (Figure 2c), and this is in agreement with the results from dynamic light scattering (DLS) measurement. The phenomenon of aggregation decreased with increasing PEG chain length to 2k and 5k (Figure 2d,e). For the PEI−PEG(20k) polyplexes, the majority of the particles was separated from each other (Figure 2f), suggesting that these polyplexes were better stabilized against agglomeration. This is in agreement with the decreased particle size measurement. Similarly, it was also reported that PEG chain length was a main determinant of polyplex aggregation (*31*).

SiRNA Condensation. To quantify siRNA condensation as a function of N/P ratio, we utilized the ability of PEI to quench siRNA/ethidium bromide fluorescence by immersing the gel in ethidium bromide solution for 5 min before imaging. If siRNA is condensed by cationic polymers, ethidium bromide can no longer intercalate, and fluorescence intensity decreases measurably. First of all, with PEI(25k)-*g*-PEG$(2k)_{10}$ copolymer taken as an example, the effect of ionic strength in the medium on the electrostatic interaction between polymer and siRNA was investigated. Complete siRNA condensation was found at N/P ratio of 5 in 150 mM NaCl solution, compared to N/P ratio of

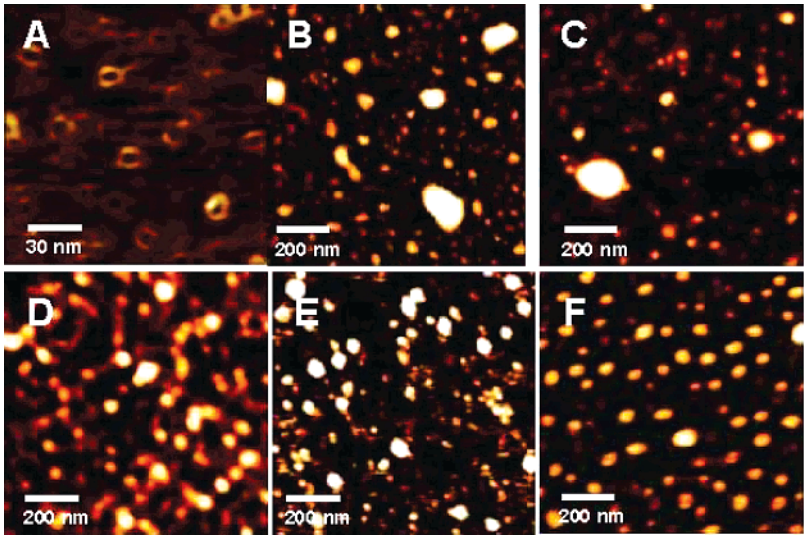


Figure 2. AFM images of different siRNA polyplexes. (a) free siRNA, (b) PEI 25kDa, (c) PEI(25k)-*g*-PEG(550)$_{30}$, (d) PEI(25k)-*g*-PEG(2k)$_{10}$, (e) PEI(25k)-*g*-PEG(5k)$_4$, (f) PEI(25k)-*g*-PEG(20k)$_1$.

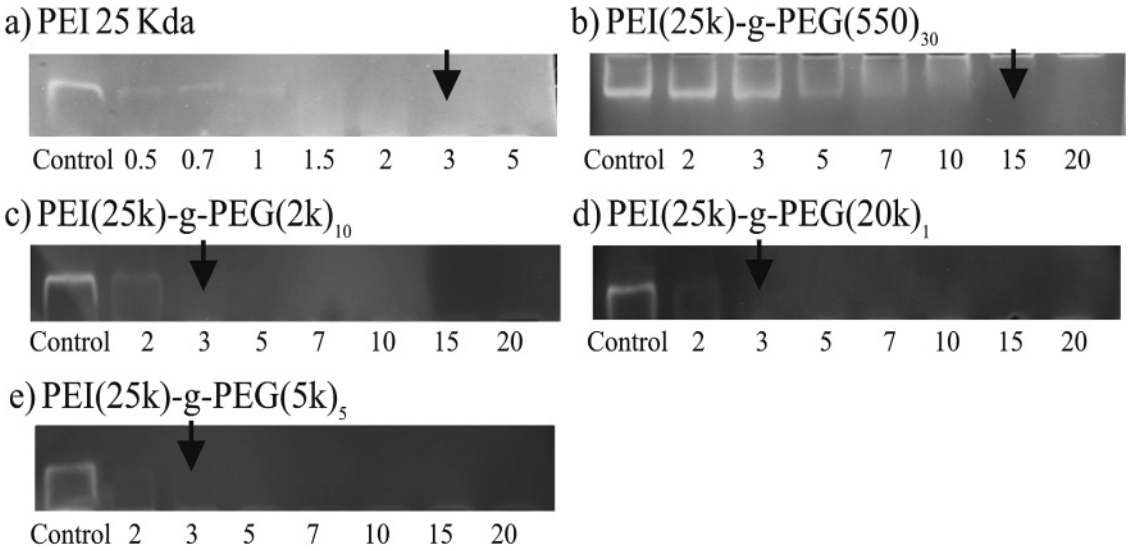


Figure 3. siRNA condensation of PEI and PEI−PEG by increasing the N/P ratio measured by polyacrylamide gel electrophoresis. Lane 1, N/P 0 (siRNA only as control); completed siRNA condensation ratio was indicated by arrows.

3 in 5% glucose solution, indicating that higher N/P is essential for complete siRNA complexation at higher ionic strength. Therefore, 5% glucose was used to prepare the polyplexes in the following studies.

As shown in Figure 3, unmodified PEI condenses siRNA even at N/P ratio as low as 0.5, and complete condensation was found at N/P ratio of 3, suggesting that all negatively charged siRNA phosphate residues are saturated with PEI. Similarly, it was reported that at N/P ratios of >2.5 plasmid DNA was fully retarded in agarose gel electrophoresis with PEI (*32*). When PEI was grafted with short PEG blocks as in the case of copolymer PEI(25k)-*g*-PEG(550)$_{30}$, the complexation was hindered slightly at N/P of 5.0 but complete condensation was realized at N/P ratio 15, probably due to the decreased charge density of the copolymer at a relatively high PEG density. In contrast, with the same copolymer, only slight hindrance for DNA complexation was found (*28*), emphasizing different properties between DNA and siRNA (*24*). No difference was found for copolymers PEI(25k)-*g*-PEG(2k)$_{10}$, PEI(25k)-*g*-PEG-(5k)$_5$, and PEI(25k)-*g*-PEG(20k)$_1$, and complete retardation of siRNA was achieved at N/P of 3, which is comparable to homopolymer PEI 25k, the same trend found in the size measurement. This implies that at similar PEG content PEG chain length plays a critical role for nucleic acid condensation. Anyway, no difference was found at PEG 2k, 5k, and 20k. In contrast, slightly increased DNA condensation was reported with increasing PEG MW from 550 to 20 kDa in the same copolymer (*28*).

Heparin Displacement. In this study, except for copolymer PEI(25k)-*g*-PEG(550)$_{30}$ (N/P 20), all the other polyplexes were prepared at N/P of 10, at which complete siRNA condensation was demonstrated in previous studies. The stability of different polymer/siRNA polyplexes against heparin displacement is shown in Figure 4. PEI/siRNA polyplexes were the most stable, and dissociation occurred at heparin 0.31 IU/μg siRNA; complete dissociation was found at heparin 0.42 IU/μg siRNA, suggesting a strong interaction of PEI with siRNA. In contrast, the stability of the polyplexes decreased after PEGylation. This observation is in agreement with our hypothesis and can probably be explained by the weakened interaction of siRNA with polymer as a result of decreased charge density. Interestingly, the protection efficiency increased with increasing PEG chain length at similar PEG content. PEI(25k)-*g*-PEG(550)$_{30}$/siRNA polyplexes were extremely unstable, and 81% siRNA dissociation was observed even at heparin of 0.10 IU/μg siRNA, which created doubt as to its capacity to bring a sufficient

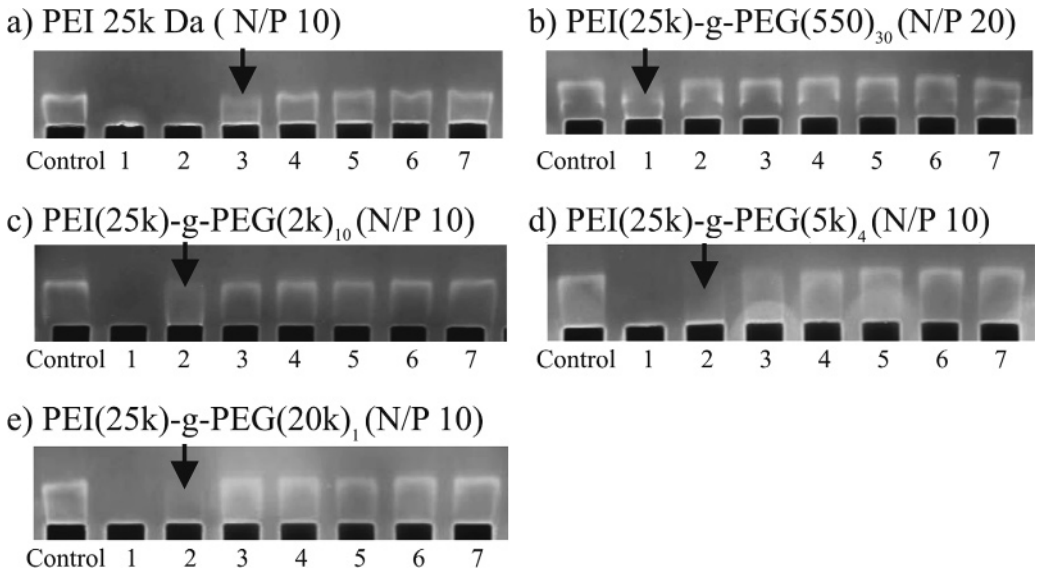


Figure 4. siRNA polyplexes stability against heparin displacement. Control: free siRNA. Lanes 1−7: heparin 0.10, 0.21, 0.31, 0.42, 0.52, 1.04, 1.57 IU/μg siRNA, respectively.

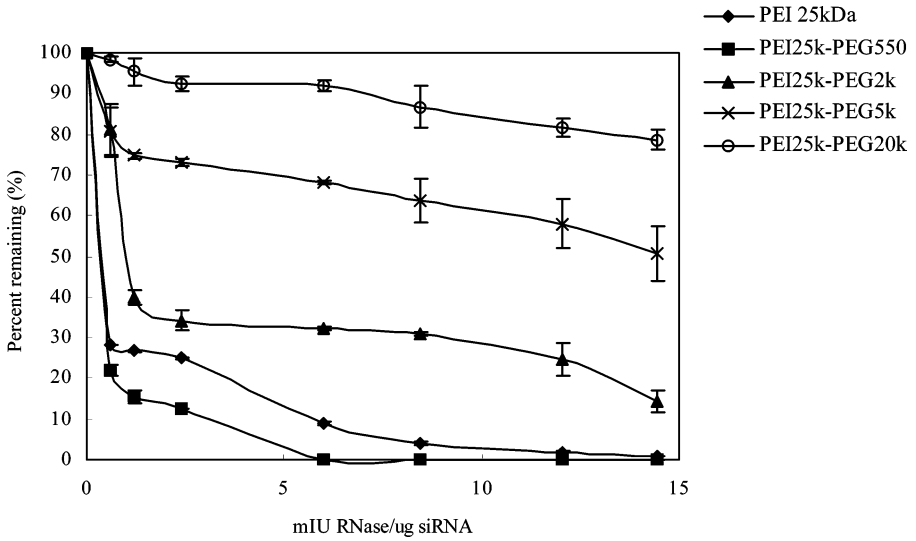


Figure 5. Incubation of PEI and PEG−PEI/siRNA polyplexes with RNase A at an N/P ratio of 10 over 0.5 h. The percentage recovery of intact siRNA is shown in reference to the intact amount of noncomplexed control siRNA.

amount of siRNA into cells. In contrast, for copolymers PEI(25k)-*g*-PEG(2k)$_{10}$, PEI(25k)-*g*-PEG(5k)$_4$, and PEI(25k)-*g*-PEG(20k)$_1$, dissociation of polyplexes occurred at heparin of 0.21 IU/μg siRNA, and 24%, 6.8%, and 5.9% of siRNA was released, respectively, indicating that PEG chain length instead of surface density played a critical role in protection of the polyplexes, and a better steric effect was found at PEG chain length of >2k. With the same copolymer PEI(25k)-*g*-PEG(2k)$_{10}$, previous study demonstrated that DNA polyplexes started to dissociate at heparin of 0.2 IU/μg DNA (*25*), which is comparable to our results. However, PEI(25k)/DNA polyplexes were less stable against heparin displacement (*25*) compared to PEG(25k)/siRNA polyplexes (0.2 IU/μg DNA vs 0.3 IU/μg DNA). This can probably be explained by the smaller MW of siRNA, which can be compacted more efficiently by the polymer. No statistically significant difference was found for the polyplexes prepared with the copolymer containing PEG 5k and 20k (P > 0.05), implying that PEG 5k might be sufficient for effective PEGylation.

Stability against RNase Digestion. The enzymatic degradation of siRNA is accompanied by a rapid decline in biological activity and therapeutic efficiency. The ability of the gene carrier to protect siRNA against enzymatic degradation by RNase, which is located in the cytoplasm, can be demonstrated by RNase digestion studies. First of all, stability of free siRNA against RNase was studied. It was found that free siRNA was degraded completely even at RNase of 0.01 mIU (0.12 mIU/μg). Thereafter, the protection effect of different PEI copolymer/siRNA polyplexes against RNase digestion was investigated. The PEG shell was expected to play an important role not only in sterically stabilizing the structure of the complex, but also in protecting the siRNA vector from being attacked by nucleases (*33*). The quantification of the siRNA displaced from the polyplexes is shown in Figure 5. At RNase of 6.0 mIU/μg siRNA, almost all the siRNA was degraded in PEI−PEG(550)/siRNA polyplexes, whereas 9% was left in PEI(25k)/siRNA polyplexes despite the strong interaction as indicated in the heparin displacement assay. In contrast, by sterically shielding the siRNA domains susceptible to proteolytic attack, the PEG layer can decrease the amount of siRNA degraded, and the effect is PEG chain-length-dependent. It showed that 32%, 68%, and 92% of siRNA was protected from degradation in PEI(25k)-*g*-PEG(2k)$_{10}$, PEI(25k)-*g*-PEG(5k)$_4$, and PEI(25k)-*g*-PEG(20k)$_1$ polyplexes, respectively. The super protection effect of PEG 5k and PEG 20k against RNase digestion suggest their promising application in vivo. The increased stability of the polyplexes

Table 2. Particle Size of Polymer/β-Gal siRNA Polyplexes in Different Media

		5% glucose		0.9% NaCl	
polymer	N/P	size	PI	size	PI
PEI 25 kDa	10	482 ± 28	0.38 ± 0.02	144 ± 12.2	0.078 ± 0.029
PEI(25k)-*g*-PEG(550)$_{30}$	20	1320 ± 4.6	0.28 ± 0.31	434 ± 55.5	0.524 ± 0.045
PEI(25k)-*g*-PEG(2k)$_{10}$	10	650 ± 65	0.46 ± 0.01	102 ± 4.96	0.273 ± 0.049
PEI(25k)-*g*-PEG(5k)$_4$	10	642 ± 24	0.47 ± 0.01	118 ± 24.1	0.320 ± 0.108
PEI(25k)-*g*-PEG(20k)$_1$	10	490 ± 85	0.45 ± 0.06	109 ± 3.86	0.270 ± 0.010

and enhanced enzymatic resistance support the hypothetical core−shell structure of these self-assembling polyplexes; that is, the siRNA is trapped within a cationic core surrounded by a hydrophilic PEG shell. The strength of the PEG shell is its chain length dependency. It is well-known that, in aqueous solutions, PEG is a highly hydrated polymer and has a high degree of segmental flexibility. It is thought to be capable of influencing the structure of several molecular layers of water, leading to its pronounced conformational entropy and large exclusion volume (*34*). The influence of molecular weight, surface density, and conformation of PEG affect dramatically the enzyme adsorption pattern. The brush conformation is presented as the highest steric protection against protein adsorption due to the steric hindrance of heavily hydrated chains of PEG molecules and their flexibility in terms of free rotation of individual polymer units around interunit linkage (*35*). The polymer chain flexibility correlates with its ability to squeeze nonlinked water molecules out of the molecular dynamic "cloud" formed over the surface (*36*). When PEG chains are short, like PEG 550, flexibility is insufficient, structured water can be "squeezed out" due to entropic instability, and RNase can adsorb on the surface of the polyplex, leading to siRNA degradation. As the molecular weight of a PEG molecule rises, the cloud temperature, which is the temperature at which the polymer separates from the water solution, also increases, suggesting an increased amount of structured water and, therefore, increased stability with increasing PEG molecular weight (*37*). As shown in our results, flexibility and high hydration of long chains like PEG 5000 and 20 000 Da allow the repulsion of RNase to a higher extent despite its low density. Similarly, computer simulations suggest that a relatively small number of molecules of a hydrophilic flexible polymer are sufficient to decrease the number of direct collisions of colloid surface with opsonizing proteins by creating a dense protective conformational cloud over the liposome surface (*38*). Oily core nanoparticles made of PLA or PEG−PLA were studied for complement consumption, and the testing of longer PEG chains (20 kDa) showed them to be more effective, even at lower surface density (*39*).

In addition, integrity of the siRNA polyplexes in the presence of RNase was investigated. No dissociation of polyplexes was found for siRNA polyplexes with PEI homopolymer and the copolymers with PEG chain lengths 2k, 5k, and 20k at N/P ratio of 10 even when the RNase amount was as high as 6 mIU/μg siRNA. In contrast, even at N/P ratio of 20, PEI(25k)-*g*-PEG(550)$_{30}$ could not inhibit siRNA release from the polyplexes even when the RNase amount was as low as 0.6 mIU/g (data not shown), implying that PEG 550 is too short to protect siRNA efficiently. Moreover, most of the PEG-modified proteins currently approved for human use by the FDA rely on PEG with MW 5000 Da or higher (*40*, *41*).

Knockdown Experiment in Cell Culture. On the basis of the in vitro properties of different polyplexes, the knockdown efficiency of siRNA polyplexes with the following copolymers, PEI(25k)-*g*-PEG(2k)$_{10}$, PEI(25k)-*g*-PEG(5k)$_4$, and PEI(25k)-*g*-PEG(20k)$_1$, were studied in cell culture and compared with that of the homopolymer PEI(25k)−siRNA polyplexes. The polyplexes were prepared in 150 mM sodium chloride solution based on the particle size data in Table 2. Lipofectamine 2000 was used as a positive control (*27*). The biological results are shown in Figure 6. All the polymers investigated could down-regulate gene expression significantly compared to the control ($P < 0.05$). Since it was reported that no difference between the use of small siRNA particles (50−100 nm) and large aggregates (200−600 nm) for gene knockdown was observed (*24*), it is reasonable to assume that the knockdown difference caused by particle size difference of the polyplexes was marginal. It was noticed that the knockdown efficiency increased with increasing PEG chain length up to PEG MW 5 k. Beyond this point, further increasing PEG MW to 20k led to no apparent change in knockdown efficiency, a similar trend observed in RNase digestion assay. The low knockdown efficiency of PEI can probably be explained by the fact that the high siRNA condensation of PEI might prevent siRNA release in the cytosol, in agreement with its high stability against heparin displacement. In contrast, for PEI−PEG polyplexes, heparin can reach siRNA in the condensed form even at quite a low concentration, implying that siRNA complexed with PEI−PEG was accessible to the targeting mRNA, in line with the enhanced knockdown efficiency. Therefore, in agreement with our hypothesis, PEGylation of PEI appears to enhance siRNA release in the cytoplasm, leading to enhanced biological activity. On the other hand, it should be mentioned that several important factors are likely to be synergistically involved in the pronounced siRNA activity of PEI−PEG(>2kDa) polyplexes, such as the improvement of the stability against enzymatic degradation, minimal interaction with serum proteins, and the effective transport of free siRNA from endosome into cytoplasm.

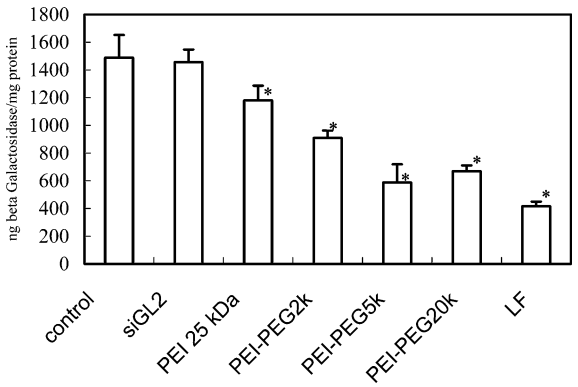


Figure 6. Biological activity of different polymer/siRNA polyplexes in NIH/3T3 fibroblasts stably expressing β-galactosidase. All polyplexes were prepared at N/P of 10. *$P < 0.05$.

Interestingly, an extremely good correlation between RNase digestion and knockdown efficiency data in cell culture was established. Figure 7 exemplifies the logarithmic relationship between stability of the polyplexes at RNase of 6 mIU and knockdown efficiency. The insert shows a linear correlation when remaining siRNA was less than 70%. It implies that RNase digestion stability could be employed as a potential tool to predict the biological activity of siRNA delivery systems.

In addition, our RNase digestion and cell culture data did indicate that stability and biological activity of siRNA polyplexes was significantly improved for the copolymer with PEG chain length of >5 kDa. This information provides further

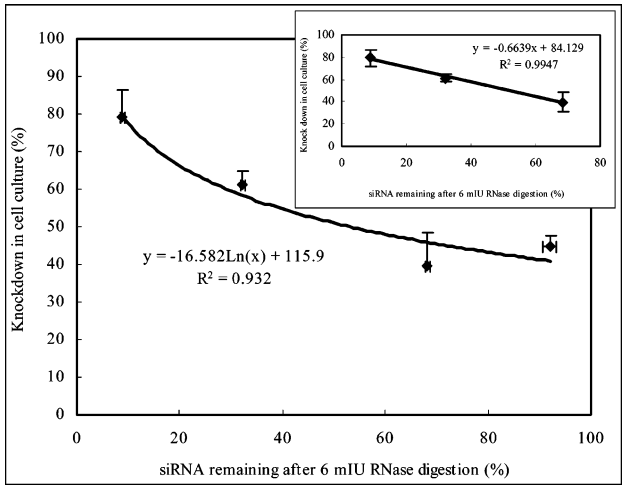


Figure 7. Correlation between RNase digestion stability and biological activity in cell culture. Insert shows a linear correlation when remaining siRNA was less than 70%.

rationale for using PEG with MW of >5 kDa in the FDA-approved protein products.

Subcellular Localization of the siRNA Polyplexes. On the basis of the results from cell culture, copolymer PEI(25k)-*g*-PEG(5k)$_4$ was selected for cellular trafficking of siRNA polyplexes. Confocal microscopy was employed to provide direct evidence for localization of polyplexes. To investigate the uptake and intracellular localization of the polyplexes, both siRNA and the copolymer were fluorescently labeled. With regard to free siRNA, rarely any red fluorescence was observed (Figure 8B). In contrast, a significantly higher internalization of siRNA was found for the copolymer/siRNA polyplexes (Figure 8A). As shown in Figure 8A, a large amount of yellow color, resulting from the colocalization of green and red, was distributed within the cytoplasm in a punctuated pattern, in small, round vesicles localized in the perinuclear area, implying that at least part of the polyplexes are being taken up by endocytosis without dissociation. The red spots in the image (Figure 8A(e)) indicated intracellular siRNA release in the cytosol after 2 h incubation. No fluorescence signal was found in the nuclear area, in this special image areas in white represent colocalization of green and red, as shown in Figure 8A(f). It shows that the polyplexes were mainly distributed in the cytosol, its site of action, instead of entering the nucleus. This is in agreement with our hypothesis. Distribution of polymer in the cells was investigated as well. Figure 8C showed that the polymer itself was dispersed throughout the cytoplasm, being taken up by the cells in an unspecific manner. This point has been mentioned in the former studies (*42*).

CONCLUSIONS

With the PEG ratio in the copolymer kept constant (~50%), influence of the polymer structure on the stability and biophysical properties of the polyplexes was studied. Interestingly, the stability and size of siRNA polyplexes were clearly influenced by PEI−PEG structure, and high degrees of substitution such as PEI(25k)-*g*-PEG(550)$_{30}$ resulted in large (300−400 nm), diffuse complexes (AFM) which showed condensation behavior only at N/P ratio of 15. All other polyplexes and the PEI control showed similar sizes (150 nm) and compact structures in AFM. Complete condensation was reached at N/P ratio of 3. Displacement of siRNA from polyplexes using heparin and stability against RNase digestion confirmed this observation. Surprisingly, protection against RNase digestion was highest for PEI(25k)-*g*-PEG(5k)$_4$ and PEI(25k)-*g*-PEG(20k)$_1$, while siRNA/PEI provided insufficient protection. In knockdown experiments

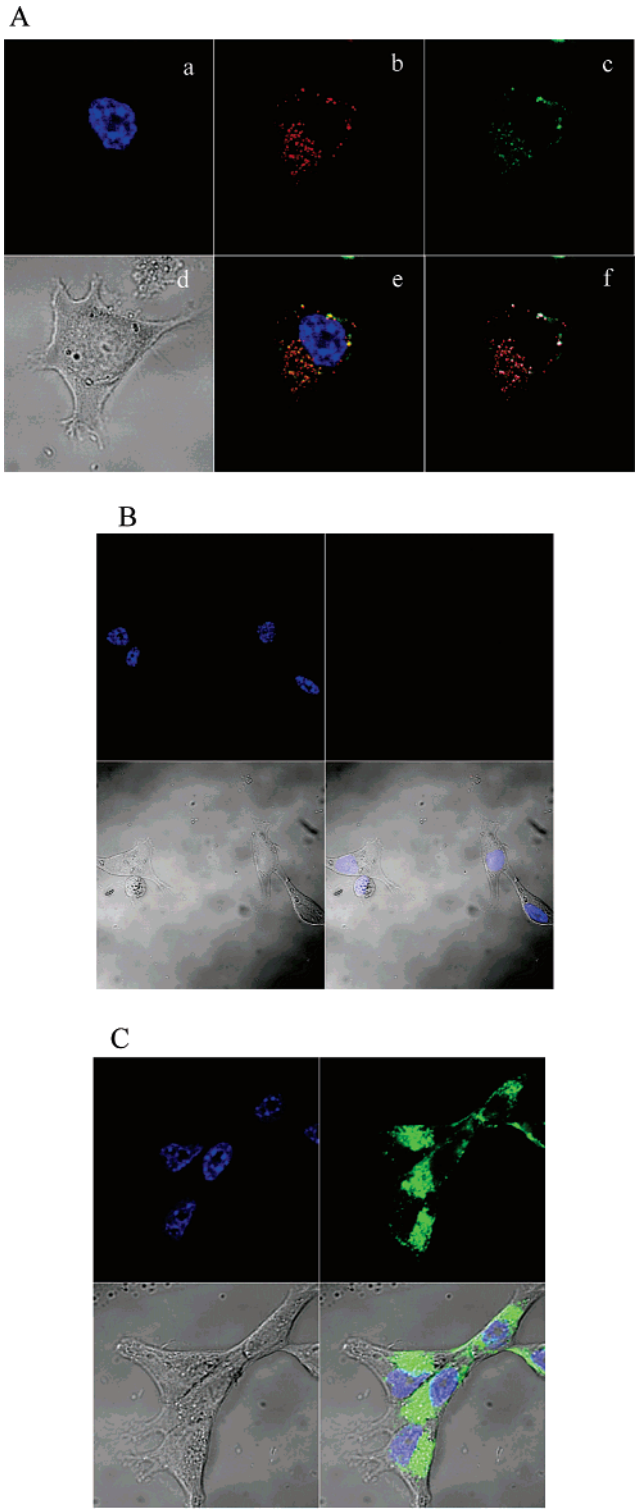


Figure 8. Confocal micrograph images of NIH/3T3 cells incubated with polyplexes, copolymer, and siRNA. Copolymer PEI(25k)-*g*-PEG(5k)$_4$ was labeled with FITC; siRNA was labeled with Cyanine 3. (A) Incubated with PEI(25k)-*g*-PEG(5k)$_4$ copolymer/siRNA polyplexes: (a) blue filter, (b) red filter, (c) green filter, (d) cell morphology, (e) overlay of a−c, and (f) overlay of b and c, indicating that the polyplexes were distributed in the cytosol instead of entering nucleus. Areas in white represent colocalization. (B) Incubated with free siRNA. (C) Incubated with free PEI(25k)-*g*-PEG(5k)$_4$ copolymer.

using NIH/3T3 fibroblasts stably expressing β-galactosidase, it was shown that PEG chain length had a significant influence on biological activity of siRNA. Polyplexes with siRNA containing PEI(25k)-*g*-PEG(5k)$_4$ and PEI(25k)-*g*-PEG(20k)$_1$ yielded similar efficiencies of ca. 70% knockdown as lipo-

fectamine controls. In conclusion, in agreement with our hypothesis, PEGylation can modify the stability of PEI/siRNA polyplexes. Release of siRNA in the cytosol depends on polyplex stability and protection against RNase digestion. A good correlation between RNase stability and knockdown efficiency in cell culture was found for the polyplexes studied. The in vivo behavior of the polyplexes is ongoing. Further optimizations are promising to develop biodegradable PEI−PEG as siRNA carriers.

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