Addition of poly (propylene glycol) to multiblock copolymer to optimize siRNA delivery

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Keywords: RNAi, siRNA delivery, copolymer, poly(ethylene glycol), poly(propylene glycol), poly(L-lysine)

Introduction

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) is an exciting, new technology with many potential biomedical applications. siRNAs are generated by cleavage of long, double-stranded RNAs (dsRNAs) by the cytoplasmic enzyme Dicer in plants and worms, but can also be synthetically produced and introduced into mammalian cells to achieve gene silencing. Inside the cells, siRNA targets specific mRNA (mRNA) for enzymatic degradation via association with the RNA-induced silencing complex (RISC). Exploiting this system, siRNAs have been designed as promising therapies to achieve gene silencing.1,2

A major challenge of using therapeutic siRNAs in vivo is effective and safe delivery. Typically, siRNAs are 7 nm in length with an approximate molecular weight of 13 KDa and have a high net negative charge. Thus, size and charge make siRNAs unable to readily penetrate cellular membranes. Furthermore, naked siRNAs are quickly degraded by nucleases in the bloodstream, and have a short half-life of minutes in the plasma. Therefore, different methods have been proposed and examined to protect and deliver siRNAs with varying degrees of success. These include use of viral vectors, cationic liposomes, and polymers. Several copolymers were designed and synthesized based on blocks of polyethylene glycol (PEG), poly(propylene glycol) PPG, and poly(L-lysine). These were designated as P1, P2, and P3. We studied the copolymer self-assembly, siRNA binding, particle size, surface potential, architecture of the complexes, and siRNA delivery. Silencing of GFP using copolymer P3 to deliver GFP-specific siRNA to Neuro-2a cells expressing GFP was almost as effective as using Lipofectamine 2000, with minimal cytotoxicity. Thus, we have provided a new copolymer platform for siRNA delivery that we can continue to modify for improved delivery of siRNA in vitro and eventually in vivo.

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Results

Synthesis of P1, P2, and P3
In this study, we designed and produced a new series of hybrid, cationic polypeptide block copolymers based on PEG, PPG, and PLL. These block copolymers were tailored for siRNA binding via amine groups in the branched side of the PLL and subsequent delivery. In this study, PEG-NH₂, H₂N-PEG-NH₂, and H₂N-PPG-PEG-PPG-NH₂ were used as initiators in the synthesis of P1, P2, and P3 polypeptide copolymers. The synthesis of the P1, P2, and P3 copolymers involved three steps as shown in Figure 1. First, LL(Z)-NCA was prepared by intramolecular ring closure of LL(Z). The hybrid copolymers, protected by Z-groups, were then synthesized using successive ring opening polymerization. After removal of the protective Z-groups on P1-Z, P2-Z, and P3-Z by HBr/HAc, target copolymers P1, P2, and P3 were obtained.

1HNMR results verified the successful synthesis of P1-Z, P2-Z, and P3-Z as shown in Figure 2. The peaks which appear at δ = 4.98 ppm and 7.22 ppm in Figure 2 correspond to the protons of the -CH₂ group in the PEG or PPG unit. The peaks that appear at δ = 4.98 ppm and 7.22 ppm represent the protons of the -CH₂ group and benzenoid group in the Cbz unit, respectively. The ratio of LL(Z) unit and the PEG or PPG unit can be obtained from 1HNMR data. As the molecular weights of the commercial PEG-NH₂, NH₂-PEG-NH₂, and NH₂-PPG-PEG-PPG-NH₂ are known, molecular weights of P1, P2, and P3 can be deduced from 1HNMR data and are shown in Table 1. P3 was achieved at N/P ratio of 2, indicating that the hydrophobic PPG blocks improve siRNA-binding (data not shown).

Polyplexes were further characterized by size and Zeta-potential measurements. Because an N/P ratio of at least 4 was needed to achieve complete siRNA condensation by all of our copolymers, all experiments discussed hereafter were performed using greater N/P ratios of 6, 12, and 24. Size and surface charge measurements are presented in Figure 4 and Figure 5, respectively. The diameters of P1 complexes were 128 nm, 130 nm, and 150 nm for respective N/P ratios of 6, 12, and 24. The diameters of P2 complexes were larger than those of P1 and P3. The two sides of PLL may contribute to making P2 complexes less compact by entrapping many water molecules in the complexes and thus, contributing to the larger

| Polymer | P1 | P2 | P3 |
|---------|----|----|----|
| Mn (kD) | 10.2 | 7.9 | 6.5 |

* Determined by 1H NMR.
complex sizes measured. It was interesting to find that increasing the N/P ratio of P1 or P2 polymer complexes resulted in larger particles, while increasing the N/P ratio of P3 polymer complexes resulted in smaller particles. Because the PPG segments are chemically attached to the PLL segments, formation of a PPG core causes dense packaging of P3-siRNA complexes. The Zeta potential analyses are provided in Figure 5. The Zeta-potentials of P1 complexes were 28 mV, 29 mV, and 31 mV for respective N/P ratios of 6, 12, and 24. The Zeta-potentials of P2 complexes range were 44 mV, 46 mV, and 52 mV for respective N/P ratios of 6, 12, and 24. Finally, the Zeta-potentials of P3 complexes were 19 mV, 20 mV, and 22 mV for respective N/P ratios of 6, 12, and 24. The Zeta-potentials of P2 complexes were greater than those of P1 and P3. It was interesting that P2 complex has greater potential but lower siRNA binding ability. A possible reason is that the larger surface areas coupled with lower siRNA binding efficiency would induce more charge leave on their surfaces. Taken together, the size and Zeta potential measurements have provided useful information on the architecture of the P1, P2, and P3 complexes: (1) P1 and P2 complexes were less dense, although they still appeared to form micelles, (2) a well-defined boundary between PLL/siRNA complexes and PEG segments was not apparent, (3) PLL segments increasingly localized to the PEG corona, and (4) P3 complex is small in size and has a relatively defined layer structure because of the PPG core. It is possible that the small Zeta potential of P3 (~20 mV) may improve its affinity to the cells and internalization by the cells as compared with the other copolymers tested. The possible micelle architecture of complexes of copolymer with siRNA is shown in Figure 6.

**Figure 2.** $^1$H NMR spectra of P1-Z, P2-Z, and P3-Z. Characterizations by $^1$H NMR using DMSO-d$_6$ as the solvent for measurements are shown.

**Evaluation of cytotoxicity by P1, P2, and P3 copolymers**

The cytotoxicity of cationic copolymers P1, P2, and P3 was evaluated by MTT viability assay in Neuro-2a cells. The cells were incubated with various concentrations of copolymers for 48 h. The results in Figure 7 show that P1 copolymers were the least toxic, while P2 copolymers were the most toxic. When treated with any of the copolymers at about 60 μg/ml, the cells maintained over 80% viability. Cells treated at concentrations of 80 μg/ml maintained over 85% viability when treated with copolymers P1 and P3, but less than 75% when treated with P2. Toxicity has been shown to be related to charge density of the polymer, and may provide an explanation for the different results observed...
Using the different copolymers. Another explanation is that toxicity might be primarily caused by addition of an excess of polymer.29

Cellular uptake of copolymers-siRNA complexes

Cellular uptake is a key step for siRNA delivery. To assess the ability of the various polymers to facilitate siRNA internalization, green fluorescent protein (GFP)-targeted siRNA labeled with a Cy3 fluorescent tag was used to form polyplexes. Fluorescence microscopy was used to monitor uptake of Cy3-tagged siRNA (red) and knockdown of GFP expression (green) in Neuro-2a/GFP cells (Fig. 8). The cells shown were transfected with polyplexes composed of the cationic copolymers P1, P2, and P3 with siRNA at N/P ratios of 6, 12, and 24. At the lowest N/P ratio of 6, red fluorescent signal was not detected inside the Neuro 2a cells and GFP expression was still very strong, indicating poor uptake of polyplexes. However, when the N/P ratio was increased to 24, labeled siRNA was detected inside the cells and GFP expression decreased. Furthermore, Cy3 signal increased while GFP expression decreased in cells treated with P2 and P3 complexes as compared with cells treated with P1 complex. Thus, at a high N/P ratio of 24, copolymers P2 and P3 demonstrated higher efficiencies for cellular uptake and gene silencing than P1.

Assessment of siRNA delivery and targeted gene silencing mediated by P1, P2, and P3 copolymers by flow cytometry

Although fluorescent microscopy results qualitatively showed major differences in siRNA delivery by the three copolymers, the ability of different polymers to enhance siRNA internalization was also quantitatively determined. Neuro-2a/GFP cells were transfected at an equivalent concentration of 80 nM siRNA complexed with the copolymers at N/P ratios of 6, 12, and 24. Lipofectamine™ 2000 was used to deliver siRNA as a positive control. The cells were treated with the complexes for 4 h and then cultured for 24 h as shown in Figure 9. For all polymers, increasing the N/P ratio resulted in decreased expression of GFP after transfection. Knockdown of GFP was not apparent when P1, P2, and P3 copolymers were complexed at N/P ratio of 6. Furthermore, P1-siRNA complexes exhibited little gene knockdown at any N/P ratio. Remarkably, the copolymers P2 and P3 coupled with siRNA led to greater gene knockdown than copolymer P1 when used at the N/P ratios of 12 and 24. The knockdown efficiencies when using copolymers P2 and P3 to deliver siRNA were similar to efficiencies observed using Lipofectamine™ 2000 lipoplexes, a standard reagent used for siRNA delivery in vitro.

Discussion

The effectiveness of our siRNA-bound complexes may be explained by their physical, structural, and chemical characteristics. P2-siRNA complex was larger in size and had a greater surface charge. A higher cationic charge in the complexes corresponds to higher transfection efficiency, and is favorable for...
intracellular release of siRNA. While this higher cationic charge of P2 complex may induce greater gene knockdown, it may also induce greater cytotoxicity. In contrast, for P3-siRNA complex, greater gene knockdown may be attributed to the PPG structure of copolymer P3 and the micelle-complex architecture of their aggregates. In addition, while copolymers P1 and P2 are water soluble block copolymers that can complex with siRNA to form micelle-like aggregates of larger size and lesser density, copolymer P3 has hydrophobic block PPG units. It is possible that the copolymer P3-siRNA complex entered the cells more readily because of its small volume and dense architecture as compared with P1- and P2-siRNA complexes.

Our results showed that copolymers P1, P2, and P3s designed were minimally toxic up to a concentration of 60 μg/ml for copolymer P2 and 80 μg/ml for copolymers P1 and P3. Furthermore, P3 was able to complex with siRNA at a low N/P ratio of 2. Compared with P1 and P2, P3 complexes are smaller and denser, and thus, may be internalized via endocytosis more readily than free siRNA, P1, or P2 complexes. P3 complexes are more efficient for siRNA delivery and gene silencing than P1. P3-siRNA complexes decreased GFP expression in GFP-expressing Neuro-2a cells to 28%. In addition to the amount of internalized amount of siRNA, the internalization pathway or endo/lysosomal escape of siRNA can also contribute to an effective RNAi effect. Of interest, siRNA delivery by P3 was similarly efficient as that by Lipofectamine™ 2000, making it a viable candidate for siRNA delivery pending further optimization.

While neither of our copolymers described outperformed the commercially available Lipofectamine™ 2000 reagent, we have provided a new copolymer platform for siRNA delivery that we can continue to modify for improved delivery of siRNA delivery in vitro and eventually in vivo.

Materials and Methods

Materials
Methoxypolyethylene glycol amine (PEG-NH₂, Mw 5000), Poly(ethylene glycol) bis(amine) (H₂N-PEG-NH₂, Mw 3400), O,O'-Bis(2-aminopropyl) polypropylene glycol-block-polyethylene glycol-block-propylene glycol (H₂N-PEG-PPG, Mw 1900), Nε-benzylxycarbonyl-l-lysine, bis(trichloromethyl) carbonate, hexane, diethyl ether, tetrahydrofuran (THF), trifluoroacetic acid (TFA), N,N-dimethylformamide, hydrogen bromide (30% in acetic acid), and other chemical reagents were purchased from Sigma-Aldrich. Lipofectamine™ 2000 transfection reagent and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies. GFP-targeted siRNAs were synthesized by Integrated DNA Technologies and the sequence used was 5′-AAGCUGACCC UGAAGUUC AdTdT-3′ for the sense strand and 5′-UGAACUUCAG GGUCAGCU UdTdT-3′ for the anti-sense strand. For cellular uptake studies, the 5′ end of the sense strand was modified with Cy3 fluorescent dye.

Synthesis and characterization of polymers
The following procedure to synthesize 3-benzylxycarbonyl-l-lysine N-carboxyanhydride (LL(Z)-NCA) was adapted from Zhou et al. Ten grams of Nε-benzylxycarbonyl-l-lysine was suspended in 100 ml of THF, the mixture was heated to 50 °C, and an equivalent amount of triphosgene was added. Three hours later, the mixture was poured into 300 ml of hexane and was stored for 16–20 h at -20 °C. Recrystallization from THF/hexane generated NCA-monomer crystals. PEG-PLL(Z) P1-Z, PLL(Z)-PEG-PLL(Z) P2-Z, and PLL(Z)-PPG-PEG-PLL(Z) P3-Z copolymers were prepared by ring opening polymerization of LL(Z)-NCA as initiated by PEG-NH₂, NH₂-PEG-NH₂, and NH₂-PEG-PPG-NH₂. Briefly, PEG-NH₂ (200 mg) and LL(Z)-NCA (500 mg) were added to 10 ml dry DMF and were stirred at 30 °C for 72 h under N₂ atmosphere. Next, the reaction was poured into an excess of diethyl ether to precipitate the...
PEG–PLL(Z) copolymer. The product was purified by repeated precipitation in diethyl ether, and then dried in a vacuum. We then proceeded with the synthesis of PEG-PLL P1, PLL-PEG-PLL P2, and PLL-PPG-PEG-PLL P3 copolymers using methods adapted from Zhou et al. with some modifications. The PEG–PLL(Z) samples were first dissolved in 5 ml of TFA. HBr (30% in acetic acid) was then added in excess and the mixtures were stirred for 5 h at room temperature. Deprotected polymers were isolated by precipitation using diethyl ether. The precipitated peptides were subsequently washed with excess diethyl ether and acetone, followed by dialysis against deionized water. The product was dried in a vacuum at –40 °C. Characterization by 1H NMR spectrum was recorded by Chemtos LLC. DMSO-d-6 was used as the solvent for 1H NMR measurements. Molecular weight (Mw) was determined from the 1H NMR data.

Determination of critical micelle concentration
Pyrene was used as a hydrophobic fluorescent probe. Different concentrations of each polymer in 0.2 ml aqueous solution were mixed with 6.7 × 10−7 M pyrene residue to measure the critical micelle concentration (CMC). The solutions were maintained at room temperature for 24 h to reach the equilibrated solubilization of pyrene in the aqueous phase. Emission spectra were recorded at 393 nm with excitation at 337 nm. Emission spectra were analyzed as a function of the polymer concentrations. The CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

Preparation and characterization of polymer-siRNA complexes
siRNA and copolymer solution were each diluted in RNase-free water or Opti-MEM (Life Technologies) at room temperature and mixed together to form complexes using different copolymer to siRNA N/P ratios or the molar ratio of cationic lipid nitrogen (N) to phosphate (P) from siRNA. The mixture was vortexed for 5 s and incubated for 30 min at room temperature before use. Lipofectamine™ 2000 reagent was used as a transfection control and Lipofectamine™ 2000: siRNA complexes were prepared as per manufacturer’s protocol.

Gel retardation assay
The ability of the copolymers for binding siRNA was evaluated by electrophoresis through 2% agarose in TAE buffer (40 mM Tris–HCl, 1 v/v% acetic acid, and 1 mM EDTA) at 100 V for 20 min. The siRNA bands were stained with ethidium bromide and imaged using a VestaDoc Imaging System (Bio-Rad Laboratories).

Particle size and surface Zeta potential
Copolymer-siRNA complexes were prepared in aqueous solution and contained 200 pmol siRNA. The size and the Zeta potential of the complexes were determined at 25 °C using the Zetasizer Nano (Malvern Instruments) Zeta potential analyzer.

Cell culture
Neuro 2a (ATCC) and Neuro 2a/GFP cells (kindly provided by Dr. N. Manjunath from Texas Tech University Health Sciences Center at El Paso) were cultured at 37 °C in 5% CO2 and 95% humidity in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. For transfection experiments, cells were cultured in complete medium with FBS, but without antibiotics.

Cytotoxicity assay
The cytotoxicity of the copolymers to Neuro 2a cell lines was assessed by an MTT viability assay adapted from Qi et al.18 The cells were seeded in 96-well plates at 6000 cells per well in 100 μl of DMEM supplemented with 10% FBS and incubated at 37 °C and 5% CO2 for 24 h. Copolymers were added at varying concentrations in a total volume of 100 μl in DMEM to the cells, which were then maintained in culture for 48 h. Next, 1 g/l of
MTT was added to each well for an incubation time of 4 h. The culture medium was then removed and 150 μL of DMSO per well was added and the samples were incubated 10 min with shaking. The absorbance was measured at 492 nm using a BioTek PowerWave XS2 microplate reader (Winooski, VT USA) with Gen5 software. Cell viability was normalized to Neuro 2a cells using a BioTek PowerWave XS2 microplate reader (Winooski, VT USA) with Gen5 software. Cell viability was normalized to Neuro 2a cells that were not treated with copolymers.

Figure 9. Gene silencing of GFP protein in Neuro 2a/GFP cells at a final concentration of 80 nM siRNA. Cells were treated with complexes of copolymer P1, P2, and P3 with GFP siRNA at N/P ratio of 6, 12, and 24. Cells were also treated with naked siRNA or lipofectamine-complexed siRNA as additional controls.

Cellular uptake
Neuro 2a/GFP cells (8 × 103 cells per well) were seeded in 12-well plates at 8 × 103 cells/well and cultured for 24 h. Then, cells were transfected with copolymer complexes containing GFP-siRNA at various N/P ratios. The final concentration of siRNA was 80 nM in each well. GFP-siRNA complexes with Lipofectamine™ 2000 was used as a control. After a 4 h incubation period, the transfection medium was discarded and supplemented with fresh medium containing 10% FBS. The cells were incubated for another 24 h. The medium was removed, and the cells were washed twice with cold PBS and detached using 0.25% trypsin-EDTA. GFP expression was quantified via flow cytometry using a Gallios flow cytometer (Beckmann Coulter) and analyzed using FlowJo software (TreeStar). The parent Neuro 2a cell line not expressing GFP was used to control for background fluorescence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
1. Kim SS, Gang G, Jodha A, Manjunath N. Staining for targeted neural delivery of siRNA in vivo. Trends Mol Med 2005; 11: 497-500; PMID:16140516; http://dx.doi.org/10.1016/j.trendsmolmed.2005.09.001
2. Srivastava R, Nangia R, Kim WJ, Petkova, in small-interfering RNA delivery. Nucleic Acid Ther 2011; 21:113-25; PMID:21774936; http://dx.doi.org/10.1038/nat.2011.289
3. Kim SS, Ahsanuzzaman S, Pour D, Shimaoka M, Shankar P. Antibody-mediated delivery of siRNAs for anti-HIV therapy. Methods Mol Biol 2011; 722:59-53; PMID:21543896; http://dx.doi.org/10.1007/978-1-61779-037-9_21
4. Tompkins SM, Le C, Yanping TM, Epstein ML. Potentiation against dextran sulfate sodium chloride by RNA interference in vivo. Proc Natl Acad Sci U S A 2004; 101:16842-6; PMID:15573705; http://dx.doi.org/10.1073/pnas.040263001
5. Biele V, Ahsanuzzaman S, Shkolnyy O, Bank S. Induction of superoxide by newly administered adenosine A1. Nat Med 2005; 11:501-3; PMID:15914430; http://dx.doi.org/10.1038/nm1614
6. Li B, Yang Q, Zhang Q, Qiu C, Xie W, Pu Q, Xu X, Liu Y, Zhang J, Wodicka DL, et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in balb/c mice. Nat Med 2005; 11:504-8; PMID:16144654
7. Schiller AM, Amati B, Xu J, Zhou L, Tang Q, Strem G, Mehlen P, Le PJ, Karin MV. Inhibitory effect of ligand-directed exosomes on innate immune responses to poly(L-lysine) and polyethylene glycol. J Am Chem Soc 2011; 133:2123-4; PMID:21431694; http://dx.doi.org/10.1021/ja112375y
8. Walter J, Warren-Klein P, Fleckhaus A, Zangemeister-Wittke U. EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed adriamycin resistant siRNA. Mol Cancer Ther 2010; 9:2674-85; PMID:20718584; http://dx.doi.org/10.1158/1535-7163.MCT-09-0412
9. Rajasekaran R, Brunton NC, Wang Z, Dyer KM, Young TD, Johnson DW, Gao GC. Patent samples of small cell carcinoma show reduced expression of TRAF1 compared with normal kidney and functional studies in vivo indicate TRAF1 promoter hypermethylation potential for targeted therapy. Pathology 2011; 44:51-8; PMID:21883954; http://dx.doi.org/10.1158/2155-9555.PATH.2011.1803
10. Zhu S, Yao TD, Yang XZ, Sun TM, Mao CQ, Song SW, Wang J. Anti-HIV single-chain antibody medi- ated PEGylated siRNA delivery for targeted breast cancer therapy. J Control Release 2012; 161:875-83; PMID:22732887; http://dx.doi.org/10.1016/j.jconrel.2012.05.015
11. Yang Y, Hu Y, Wang Y, Li J, Lie F, Huang L. Nanoparticle Delivery of Pooled siRNA for Effective Treatment of Non-Small Cell Lung Cancer. Mol Pharms 2012; (Forthcoming); PMID:22605956; http://dx.doi.org/10.1021/mp300152v
12. Kola I, Krewer AR, Altmann S. RNA therapeutics: beyond RNA interference and antisense oligonu- cleotides. Nat Rev Drug Discov 2012; 11:129-30; PMID:22622536
13. Castaño D, Bollag J. The promises and pitfalls of RNA-interference-based therapeutics. Nature 2009; 457:42-3; PMID:19354783; http://dx.doi.org/10.1038/nature07770
14. Nie M, Fischer O, Kinz TF. Recent advances in targeting gene transfer vector design based on poly(DL-lactide-co-glycolide) and its derivatives. J Gene Med 2005; 7:1002- 1010; PMID:15929783; http://dx.doi.org/10.1002/jmg.20377
15. Alba CH, Chan SY, Bao TH, Kin SE. Synthesis of biodegradable multiblock copolymers of poly(DL- lactic acid) and polyethylene glycol as a non-viral gene carrier. J Control Release 2014; 197:567-74; PMID:25211887
16. Salooja EE, Wagner E. Chemically programmed polyplexes for targeted DNA and siRNA transfection. Top Curr Chem 2010; 296:227-49; PMID:21949246; http://dx.doi.org/10.1007/978-1-61779-037-9_21, 2010; 09
17. Fukusaki M, Miyata M, Muthusamy S, Kataoka K. Magnetically targeted polyplexes made from triblock copolymers with spatially ordered layering of conditional polyDEA and cathelicidin units for enhanced intracellular gene delivery. J Am Chem Soc 2012; 134:16486-9; PMID:23150909; http://dx.doi.org/10.1021/ja303440g

36 Bioengineered Volume 5 Issue 1
18. Qi R, Liu S, Chen J, Xiao H, Yu L, Huang Y, Jing X. Biodegradable copolymers with identical cationic segments and their performance in siRNA delivery. J Control Release 2012; 159:251-60; PMID:22285552; http://dx.doi.org/10.1016/j.jconrel.2012.01.015

19. Stanek V, Arini Y, Richard D, Affolter C, Nguyen J, Crucia C, Schulte P, Barbe G, Fuchs R, Oger J. Fluorescence quantification of DNA (induced by methyl phospholipids) and visualization efficiency. Biomacromolecules 2006; 9:2048-55; PMID:16973129; http://dx.doi.org/10.1021/bm601087b

20. Sato A, Cho SW, Hara M, Yanase A, Mizutani R, Yano T, Takagi M, Kato A, Shimamizu A, Mitamura A. Polyethylene imine-stabilized polyplex for the siRNA carrier with long circulation half-life. J Control Release 2007; 122:205-10; PMID:17343499; http://dx.doi.org/10.1016/j.jconrel.2007.04.018

21. Moham S, Wohrer P, Davis MF. Effects of glucosyl residues on the cellular uptake and intracellular trafficking of non-viral gene delivery particles. Eur J Cell Biol 2004; 83:97-103; PMID:15245306; http://dx.doi.org/10.1078/0171-9335-004036

22. Qiu JS, Rui YP. Self-assembled polyethyleneimine-graft-poly(epsilon-caprolactone) micelles as potential dual carriers of genes and anti-cancer drugs. Biocatalysis 2007; 28:43-52; PMID:17784425; http://dx.doi.org/10.1016/j.biocat.2007.03.015

23. Swarnkar RR, Strizeman SK, Narayan-G, Brown S, Dalby RA, Trechkin VP. Polyethyleneimine-lipid conjugate-based pH-sensitive micelle carrier for gene delivery. Biomacromolecules 2012; 13:3942-51; PMID:22939093; http://dx.doi.org/10.1021/bm300989v

24. Tian HY, Dong C, Liu H, Sun J, Deng M, Chen X, Jing X. Biodegradable cationic PEI/PEO/PLG hyperbranched block copolymers: synthesis and micelle characterization. Biomacromolecules 2005; 6:4263-71; PMID:16096163; http://dx.doi.org/10.1021/bm050212v

25. Ma CQ, Da ZY, Tan TM, Yang YN, Song DF, Wang J. A biodegradable amphiphilic and cationic triblock copolymers for the delivery of siRNA targeting the acid ceramidase gene for cancer therapy. Biomaterials 2011; 32:1024-30; PMID:21277816; http://dx.doi.org/10.1016/j.biomaterials.2011.01.006

26. Shama R, Lee JS, Bumcrot RC, Xiao C, Konnunci S, Wang YF. Effects of the incorporation of a hydrophilic middle block into a PEI-polycation-siRNA complex on the physicochemical and cell interaction properties of the polycation-DNA complex. Biomacromolecules 2009; 10:3264-71; PMID:19643877; http://dx.doi.org/10.1021/bm900876e

27. Li YF, Hua X-H, Xiao W, wang H-Y, Liu X-J, Li C, Cheng S-X, Zhang X-Z, Zhu B-X. Dual vectors of anti-cancer drugs and genes based on pH-sensitive micelles self-assembled from hybrid polycationic copolymers. J Mater Chem 2011; 21:1010-6; http://dx.doi.org/10.1039/c0jm03985y

28. Alcon MH, Green MD, Caron MA, Miller KM, Long TE. Tailoring charge density and hydrogen bonding of imidazolium derivatives for efficient gene delivery. Biomacromolecules 2011; 12:2243-50; PMID:21375069; http://dx.doi.org/10.1021/bm101603k

29. Vadeer P, van der Aa LJ, Engbersen JF, Beting J-G, Schillers HA. Dendritic-based poly(amine-amino) for siRNA delivery: effects of structure on siRNA complexation, cellular uptake, gene silencing, and toxicity. Pharm Res 2011; 28:1013-22; PMID:21885466; http://dx.doi.org/10.1007/s11095-010-0944-y

30. Zhou C, Qi X, Li P, Chan WY, Mandl L, Chang MW, Lou PL, Chan-Park MB. High potency and broad-spectrum antimicrobial peptides synthesized via incorporating polymers of alphan-carboxylic acids. Biomacromolecules 2010; 11:607-15; PMID:19557892; http://dx.doi.org/10.1021/bm900986h