

Multifunctional Triblock Nanocarrier (PAMAM-PEG-PLL) for the Efficient Intracellular siRNA Delivery and Gene Silencing

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Design and creation of novel nanometer-size carriers for the safe delivery of small interfering RNA (siRNA) toward their potential applications in cancer therapy is one of the challenging and rapidly growing areas of research. RNA interference (RNAi) is a conservative biological response to siRNA that regulates the expression of protein coding genes.^{1–5} However, the broad therapeutic applications of siRNA are limited by major delivery problems.⁶ The efficient *in vivo* gene knock down requires a delivery system that would overcome the following limitations: (1) low cellular uptake, (2) poor endosomal escape, (3) substantial liver and renal clearance, (4) facile enzymatic degradation in the blood and extracellular environment, and (5) inefficient gene silencing.

Recent investigations in the area of nanomaterials for RNA delivery, including the works in our laboratory, provided solutions to some of the major siRNA delivery problems.^{7–16} However, the developed delivery approaches address only selected siRNA delivery problems lacking optimal balanced delivery system that includes a solution for all the major aforementioned challenges. For example, a biodegradable polymer poly-L-lysine (PLL) is being used for gene delivery, and its polyplexes are taken up into cells efficiently. However, transfection efficiencies of PLL–siRNA complexes remain several orders of magnitude lower when compared that of other transfection agents. One potential reason for inefficient transfection has been identified as the lack of amino groups with a $pK_a \sim 5–7$ for so-called “proton sponge effect” that offers endosomolysis and subsequent release of siRNA. The desired transfection effect was achieved by structural modification of PLL using a targeting

ABSTRACT A novel triblock poly(amido amine)-poly(ethylene glycol)-poly-L-lysine (PAMAM-PEG-PLL) nanocarrier was designed, synthesized, and evaluated for the delivery of siRNA. The design of the nanocarrier is unique and provides a solution to most of the common problems associated with the delivery and therapeutic applications of siRNA. Every component in the triblock nanocarrier plays a significant role and performs multiple functions: (1) tertiary amine groups in the PAMAM dendrimer work as a proton sponge and play a vital role in the endosomal escape and cytoplasmic delivery of siRNA; (2) PEG, a linker connecting PLL and PAMAM dendrimers renders nuclease stability and protects siRNA in human plasma; (3) PLL provides primary amines to form polyplexes with siRNA through electrostatic interaction and also acts as penetration enhancer; and (4) conjugation to PEG and PAMAM reduced toxicity of PLL and the entire triblock nanocarrier PAMAM-PEG-PLL. The data obtained show that the polyplexes resulted from the conjugation of siRNA, and the proposed nanocarriers were effectively taken up by cancer cells and induced the knock down of the target *BCL2* gene. In addition, triblock nanocarrier/siRNA polyplexes showed excellent stability in human plasma.

KEYWORDS: siRNA delivery · PAMAM dendrimer · BCL2 silencing · siRNA stability

ligand or endosomolytic agents such as chloroquine or fusogenic peptides.^{17,18} A significant improvement in transfection efficiency was observed when histidine or imidazole moieties were attached to the PLL.^{19,20}

Another major challenge in the safe delivery of siRNA is its facile enzymatic degradation in cytoplasm due to the presence of nucleases that dramatically reduce siRNA half-life. Previously, we have reported that internally quaternized and cancer-targeted polyamidoamine (PAMAM) dendrimers provide for the efficient cellular uptake and excellent gene silencing.¹⁴ It was shown that surface modification and internal quaternization of dendrimers reduced their cytotoxicity and substantially improved the cellular uptake, while targeting of the dendrimers to cancer cells initiated receptor-mediated endocytosis and led to the efficient gene knock down. We have also demonstrated the importance of free tertiary amine groups

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in dendrimers for endosomal escape. The present study explores a different approach and is aimed at the design, synthesis, and evaluation of a triblock delivery system that provides solutions for major problems in siRNA delivery, such as poor cellular uptake, low endosomal escape, and facile enzymatic degradation. A novel triblock nanocarrier, PAMAM-PEG-PLL, has been designed to combine individual features of PAMAM dendrimer, polyethylene glycol (PEG), and poly-L-lysine. PAMAM dendrimer provides tertiary amines for endosomal escape; PEG covers up siRNA, protecting it from enzymatic degradation, while PLL offers cationic amine groups for electrostatic interaction with negatively charged siRNA.

RESULTS AND DISCUSSION

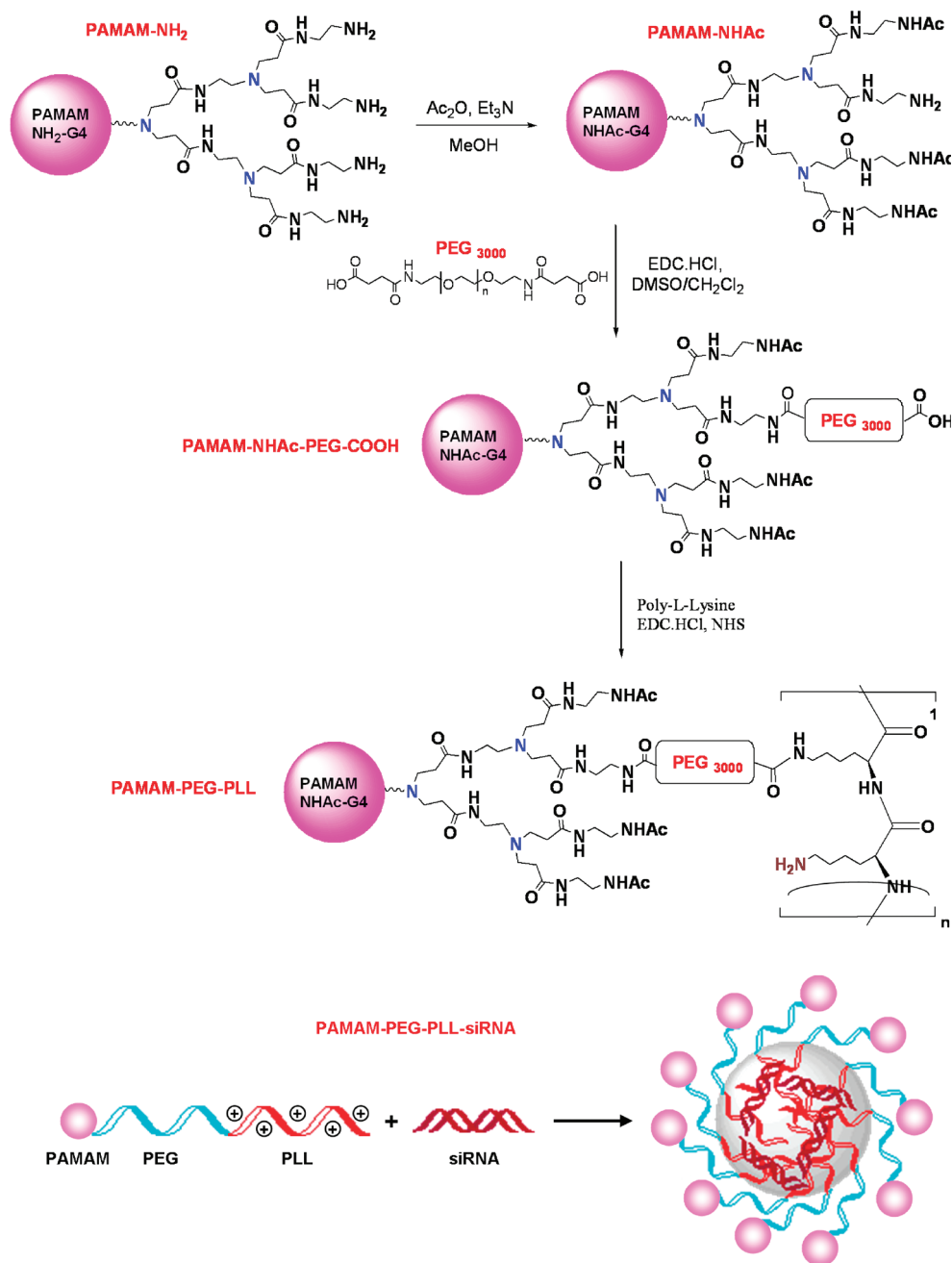
Delivery of siRNA into the cytoplasm of cancerous cells where it triggers sequence-specific mRNA degradation has recently emerged as a powerful tool in gene therapy. The major obstacles in safe transportation of siRNA have been extensively investigated, and condensation to nanoparticles has now been recognized as the most efficient method for facile transport of siRNA. Therefore, designing a nanocarrier that enables effective and safe transfer of siRNA into mammalian cells is a task of great interest. We are actively involved in the design, synthesis, and evaluation of various multifunctional nanoparticles as siRNA delivery carriers that include liposomes, surface-modified dendrimers, mesoporous silica nanoparticles, and surface-engineered superparamagnetic iron oxide (SPIO) nanoparticles, *etc.*^{7,11,13,14,16,21–25} In general, combining multiple functions in a single delivery system is a difficult task and requires laborious synthetic efforts. In continuation with our previous studies on developing multifunctional nanomedicines, herein we describe the design, synthesis, and evaluation of a synthetically simple yet novel triblock multifunctional nanocarrier, PAMAM-PEG-PLL, that effectively combines three functionalities which are otherwise ineffective when tested individually. The triblock nanocarrier PAMAM-PEG-PLL serves three distinct functions: (1) PLL provides cationic primary amine groups for electrostatic interaction with negatively charged siRNA; (2) PAMAM dendrimer offers necessary tertiary amine groups for proton sponge effect; while (3) PEG confers nuclease stability in blood serum.

A three-step synthetic route was used for the preparation of PAMAM-PEG-PLL nanocarrier (Scheme 1). In the first step, PAMAM dendrimer was partially acetylated to afford PAMAM-[(NHAc)₅₈(NH₂)₆] dendrimer. The second step involved synthesis of PAMAM-PEG-COOH by reacting one of the acid groups of α,ω -bis-(2-carboxyethyl)polyethylene glycol ($M_w = \sim 3000$) with one of the primary amines of the PAMAM-[(NHAc)₅₈(NH₂)₆] dendrimer. One could expect that the length of the PEG block would have great influence on the final

triblock–siRNA complex as a shorter length might not be enough to protect siRNA from the enzymatic degradation. However, the exact length of PEG required to achieve efficient siRNA protection is unknown. Instead of using a single long PEG chain length, we used medium size chain ($M_w = 3000$), while the ratio of triblock nanocarrier/siRNA was taken as 3 (N/P = 3). Thus, a single siRNA is surrounded by three PEG blocks and expected to give effective protection. Previously, we tested the cellular penetration of PEG with different molecular weight (up to $M_w = 20000$ Da) and found that PEG with M_w around 3000 Da penetrates cells faster when compared with higher M_w polymers.²⁶ On the basis of these considerations, we selected PEG with $M_w = 3000$ Da for these studies. During the third step, the terminal free acid group of PAMAM-PEG-COOH was reacted with PLL using EDC as a coupling reagent.

One can assume that the synthetic procedure used could not be selective and the design led to some cross-linking reactions. However, we are certain that our synthetic scheme can be selective because it is a sequential procedure and the following reasons contribute to the selective formation of PAMAM-PEG and not a PAMAM-PEG-PAMAM cross-linking polymer: (1) PAMAM-NHAc has only 5–6 free amines to react with the PEG-dicarboxylic acid; (2) the free amine groups on the dendrimer are crowded with NHAc substituent and not easily available for cross-linking; (3) dilution of reaction also plays an important role in reducing the cross-linking reaction; (4) this reaction greatly differs from the usual small molecules where selectivity would be an issue; (5) in the present studies, more harsh conditions, prolonged reaction time, and more equivalents of coupling reagents would be required to force the cross-linking PAMAM-PEG-PAMAM reaction; (6) we followed the usual technique of filtering the reaction mixture after coupling reaction to remove any insoluble material; however, in this reaction, negligible amount of insoluble material was formed; (7) even if the cross-linking product is formed, it was in trace amounts and removed completely during filtration. The coupling reaction of PAMAM-PEG with PLL can be selective to form the desired PAMAM-PEG-PLL as PLL has several amine groups available to react in comparison with 5–6 amine groups of the PAMAM dendrimer. The ¹H NMR was very useful and informative in determining the content of the nanocarrier. The area under the peak would greatly vary (almost double or half) if the number of units (PAMAM, PEG, and PLL) changes in the final nanocarrier. Each block has a large number of protons and cannot be ignored.

Theoretically, there might be a possibility that some molecules of siRNA could form complexes directly with the PAMAM dendrimer, not with positively charged PLL. The PAMAM dendrimer used in the present study was surface-modified and lacks free primary amine groups required to form a complex with siRNA. As ¹H



Scheme 1. Synthesis of triblock PAMAM-PEG-PLL nanocarrier.

NMR studies show, very few (~ 5 – 6) free amines are available on the PAMAM dendrimer. Such an amount is not enough to form complexes with siRNA that possess about 42 negatively charged phosphate groups. Our previous studies on quaternized QPAMAM–OH dendrimers suggested that tertiary amines in the PAMAM dendrimer do not participate in direct complexation with siRNA, and a stable complex is formed only after quaternizing the internal tertiary amine groups.¹⁴ This supports the fact that the PAMAM dendrimer would not form a complex with siRNA.

We attempted to determine the molecular weight of the nanocarrier by MALDI-TOF; however, even after

several trials, the triblock nanocarrier did not show clear mass. The following three matrix compounds were used to record MALDI-TOF: sinapinic acid, α -cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid. More than likely, the triblock nanocarrier did not ionize under the attempted conditions. All proton NMR spectra were recorded in solution of studied compounds in D_2O using a 400 MHz NMR spectrometer. The chemical shift (δ) was expressed as parts per million (ppm). The data obtained confirmed the structures of synthesized substances. PAMAM-NHAc 1H NMR spectral data are shown in Figure 1a. The following peaks were identified: δ 1.98 (s, $COCH_3$), 2.40–2.50

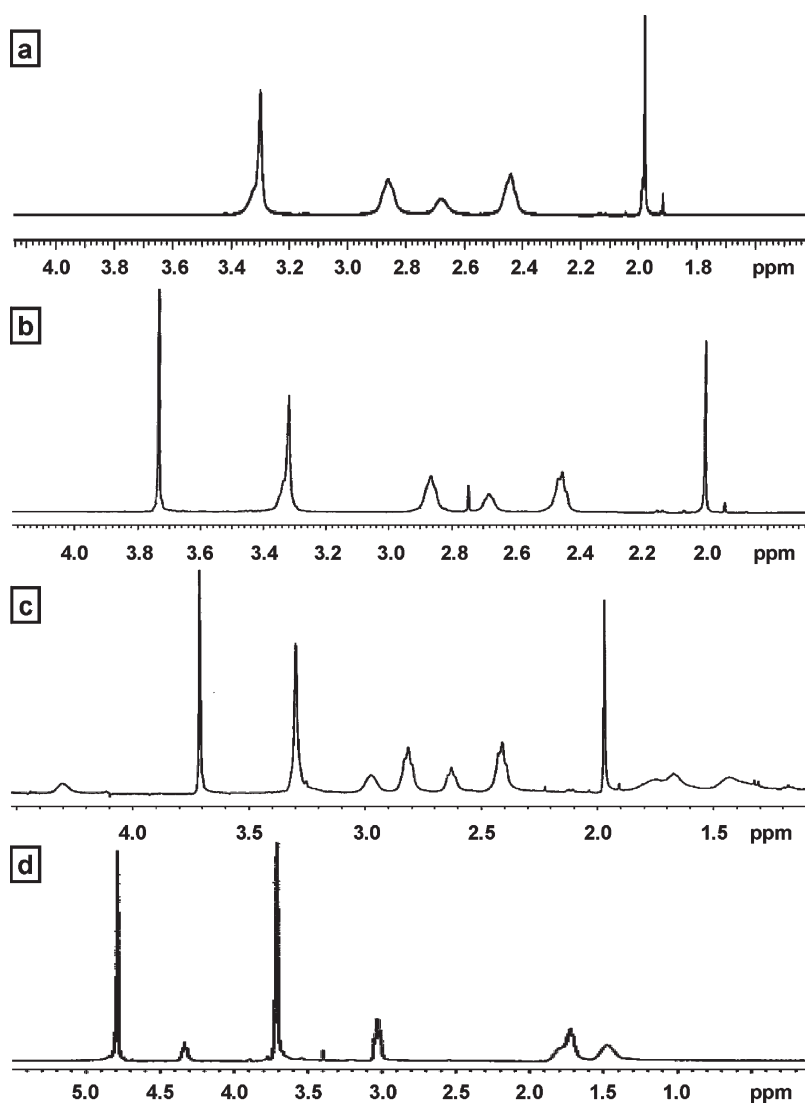


Figure 1. Representative ^1H NMR spectra in (a) D_2O of PAMAM-NHAc; (b) PAMAM-PEG-COOH; (c) PAMAM-PEG-PLL; (d) PLL-PEG-OMe.

(br m, CH_2CONH), 2.62–2.73 (br m, $\text{CONHCH}_2\text{CH}_2\text{N}$), 2.82–2.92 (br m, $\text{NCH}_2\text{CH}_2\text{CONH}$), 3.26–3.37 (m, CONHCH_2 and $\text{CH}_2\text{NHCOCH}_3$). The degree of acetylation ($\sim 90\%$) was confirmed from the proton NMR spectra (Figure 1a) and by calculation, the ratio between the integrated peak area of signal appeared at δ 1.98 ppm ($-\text{NHCOCH}_3$) to that of methylene protons of the PAMAM dendrimer (δ 2.40–3.37). PAMAM-PEG-COOH ^1H NMR spectrum (Figure 1b) showed the following peaks: δ 2.00 (s, COCH_3), 2.40–2.50 (br m, CH_2CONH), 2.64–2.75 (br m, $\text{CONHCH}_2\text{CH}_2\text{N}$), 2.82–2.92 (br m, $\text{NCH}_2\text{CH}_2\text{CONH}$), 3.30–3.38 (m, CONHCH_2 and $\text{CH}_2\text{NHCOCH}_3$), 3.74 (s, $-\text{CH}_2\text{CH}_2\text{O}$). The spectra showed monoacylation of the dendrimer with α,ω -bis-(2-carboxyethyl)polyethylene glycol, leaving another $-\text{COOH}$ group free for conjugation with poly-L-lysine (PLL). The ^1H NMR spectra for this compound confirmed the presence of both polyethylene glycol and dendrimer protons. Further monofunctionalization was determined by calculating the area under the

proton peaks arising from polyethylene glycol ($M_w = \sim 3000$, $-\text{CH}_2\text{CH}_2\text{O}-$, ~ 270 H) and dendrimer ($-\text{COCH}_3$, 174 H) appeared at δ 3.74 and δ 2.00, respectively. PAMAM-PEG-PLL ^1H NMR spectral data are shown in Figure 2c. The following peaks were identified: δ 1.30–1.50 (br m, PLL), 1.60–1.90 (br m, PLL), 1.98 (s, PAMAM), 2.40–2.50 (br m, PAMAM), 2.60–2.70 (br m, PAMAM), 2.78–2.88 (br m, PAMAM), 2.90–3.05 (br m, PLL), 3.26–3.37 (m, PAMAM), 3.70 (s, PEG), 4.25–4.35 (br m, PLL). The spectra confirmed the formation of the PAMAM-PEG-PLL nanocarrier showing the presence of proton peaks arising from dendrimer (PAMAM), polyethylene glycol (PEG), and poly-L-lysine (PLL). PEG-PLL ^1H NMR spectrum (Figure 1d) showed the following peaks: δ 1.38–1.60 (br m, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$), 1.68–1.90 (br m, $-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CO})\text{NH}-$ and $-\text{CH}_2-\text{CH}_2-\text{CH}_2\text{NH}_2-$), 2.90–3.10 (br m, $-\text{CH}_2-\text{CH}_2\text{NH}_2-$), 3.70 (s, $-\text{CH}_2\text{CH}_2\text{O}$ PEG), 4.30–4.40 (br m, $-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CO})\text{NH}-$). ^1H NMR technique has been widely used and documented as one of the best methods for

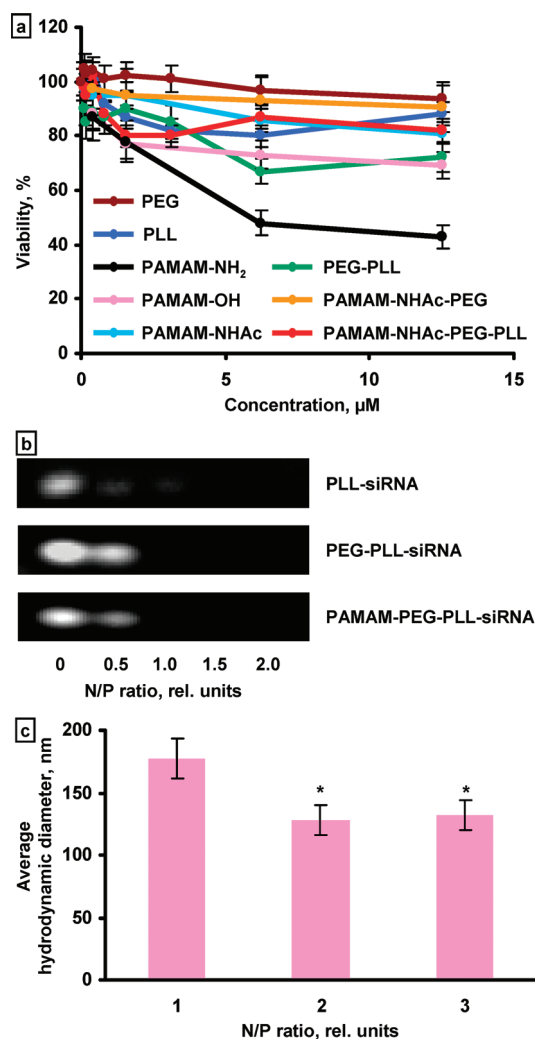


Figure 2. Characterization of different nanocarriers and their complexes with siRNA: (a) viability of human cancer cells incubated with carriers indicated (means \pm SD are shown); (b) representative images of agarose gel electrophoresis of siRNA complexes with different carriers; (c) average hydrodynamic diameter of PAMAM-NHAc-PEG-PLL-siRNA complexes formed at different N/P ratio. Means \pm SD are shown. * $P < 0.05$ when compared with N/P ratio equal to 1.

calculating the functional group content of dendrimers. The number of amine groups left free after acetylation reaction can be calculated using the ^1H NMR technique.²⁷ Since the triblock nanocarrier is a combination of linear as well as spherical polymer molecules, it will not follow the behavior of the conventional polymer molecules, and therefore, the conventional molecular weight determination method based on calibration cannot be used in such cases. More studies and special setups are required to use the gel permeation chromatography (GPC) method to determine the molecular weight. We found that ^1H NMR spectroscopy was highly useful and provided the desired information by comparing the area under the peak. The number of free amines in the PAMAM-PEG-PLL nanocarrier was calculated based on the degree of polymerization in

the poly-L-lysine polymer. There could be a very small variation in the calculated and actual content of the functional group in the nanocarrier; however, we believe that it would not greatly affect its function. Gel electrophoresis studies also provided indirect evidence on the accuracy of calculated content of the functional group in triblock nanocarriers. The band for siRNA completely disappeared when the ratio of N/P was 1; that is, the number of cationic amines/phosphate of siRNA is 1/1. If the calculated amount of cationic amines is not correct, the nanocarrier/siRNA complex formation can be expected at a different N/P ratio ($-\text{NH}_2$ of nanocarrier/phosphate of siRNA).

The measurement of viability of cells incubated with different concentrations of PEG, PLL, PEG-PLL, PAMAM-NHAc-PEG, and PAMAM-NHAc-PEG-PLL compounds showed their relatively low cytotoxicity (Figure 2a). No substantial differences were found between different nanocarriers under the concentrations of the compounds lower than $4 \mu\text{M}$. However, the concentrations exceed $4 \mu\text{M}$, PEG-PLL demonstrated higher cytotoxicity when compared with PLL alone and PAMAM-NHAc-PEG-PLL. Therefore, the toxicity of PEG-PLL was reduced when PEG-PLL was conjugated to the PAMAM-NHAc dendrimer. Cytotoxicity data for the triblock PAMAM-NHAc-PEG-PLL nanocarrier were compared with cytotoxicity of its previously synthesized predecessors (PAMAM-NH₂, PAMAM-OH, and PAMAM-NHAc dendrimers).¹³ The results of such comparison showed a comparably low cytotoxicity for triblock PAMAM-NHAc-PEG-PLL and acetylated PAMAM-NHAc dendrimers. The cytotoxicity under the high concentrations of both dendrimers was lower when compared with quaternized the non-acetylated PAMAM-OH dendrimer. In contrast, nonmodified PAMAM-NH₂ dendrimer demonstrated a significant cellular toxicity under concentrations higher than $5 \mu\text{M}$. It should be stressed that a maximum decrease in viability of cells incubated with PEG, PLL, PEG-PLL, PAMAM-NHAc-PEG-PLL, PAMAM-OH, and PAMAM-NHAc compounds was substantially higher than 50% under all studied concentrations. Such low toxicity does not allow calculating the IC_{50} dose for these substances (half-maximal inhibitory concentration, the dose that kills about 50% of cells). In contrast, the IC_{50} dose of nonmodified PAMAM-NH₂ dendrimer was estimated to be around $6 \mu\text{M}$ (Figure 2a).

The nanocarrier-siRNA complex formation and optimal N/P ratio was determined by agarose gel electrophoresis. The PLL, PEG-PLL, and PAMAM-PEG-PLL nanocarriers were mixed with siRNA in water at various N/P charge ratios and were subjected to electrophoresis in agarose gel (Figure 2b). The numbers of cationic primary amine groups in PLL, PEG-PLL, and PAMAM-PEG-PLL were calculated based on PLL M_w (~ 8000) and degree of polymerization (57). All three nanocarriers showed the complex formation at the N/P ratio of 1 and

above as evidenced by oligonucleotide band disappearance from agarose gels. The quantitative analysis showed that fluorescence of PLL-siRNA, PEG-PLL-siRNA, and PAMAM-PEG-PLL-siRNA progressively decreased with the increase in N/P ratio. For PLL-siRNA, the complexation decreased fluorescence to 19, 8, and 0% with N/P ratio equal to 0.5, 1.0, and 1.5 relative units, respectively; for PEG-PLL-siRNA, fluorescence decreased to 19, 8, and 0% with N/P ratio equal to 0.5, 1.0, and 1.5 relative units, respectively; for PAMAM-PEG-PLL-siRNA, fluorescence decreased to 45 and 0% with N/P ratio equal to 0.5 and 1.0, respectively.

The hydrodynamic diameter of the PAMAM-PEG-PLL-siRNA complex was determined by dynamic light scattering at a charge ratio ranging from 1 to 3 relative units. The PAMAM-PEG-PLL/siRNA particle size slightly decreased by increasing the charge ratio to 3 relative units (Figure 2c). The measurements of zeta-potential of dendrimer-siRNA complexes showed that the complexes were neutral. The size of the PAMAM-PEG-PLL-siRNA complexes used in this paper varied from 120 to 180 nm depending on N/P ratio. This relatively large size is attributed to cross-linking of nanocarrier and siRNA. Previously, we have shown that an internally charged dendrimer gives small, compact, and spherical nanoparticles with siRNA; however, commercial PAMAM-NH₂ showed nanofibers due to cross-linking.²⁶ In the present experiments, we expect a similar trend because PLL possesses primary amine groups.

The cellular uptake of naked and complexed fluorophore-labeled siRNA (siGLO Red, red fluorescence) was studied in living (not washed and fixed) cells using confocal microscopy. A2780 human ovarian cancer cells were incubated with free siRNA and PAMAM-PEG-PLL-siRNA complex and were subjected to confocal microscopy. Consistent with our previous findings,^{13,14} naked siRNA did not penetrate the cancer cells (Figure 3a). Previously, we have reported that PAMAM-NH₂ and PAMAM-OH dendrimers failed to deliver siRNA into cells, while the acetylation of the PAMAM dendrimer surface substantially improved internalization of PAMAM-siRNA complexes.¹³ On the basis of this finding, we used a PAMAM dendrimer with the acetylated surface further modified with PEG and PLL. It was found that siRNA complexed with a PAMAM-PEG-PLL cationic nanocarrier provided excellent cellular uptake (Figure 3b). Moreover, optical section z-series of a single living cell showed the homogeneous and uniform distribution of siRNA-dendrimer complex in different cellular layers from the top of the cell to the bottom (Figure 3c). We would like to stress again that all experiments were performed on living cells without staining, fixation, and washing out the media with fluorescently labeled siRNA-dendrimer complexes. Because of this, one can see red fluorescence inside both the cells and media. In contrast, naked siRNA could be seen just in the media but not in the cell (Figure 3a).

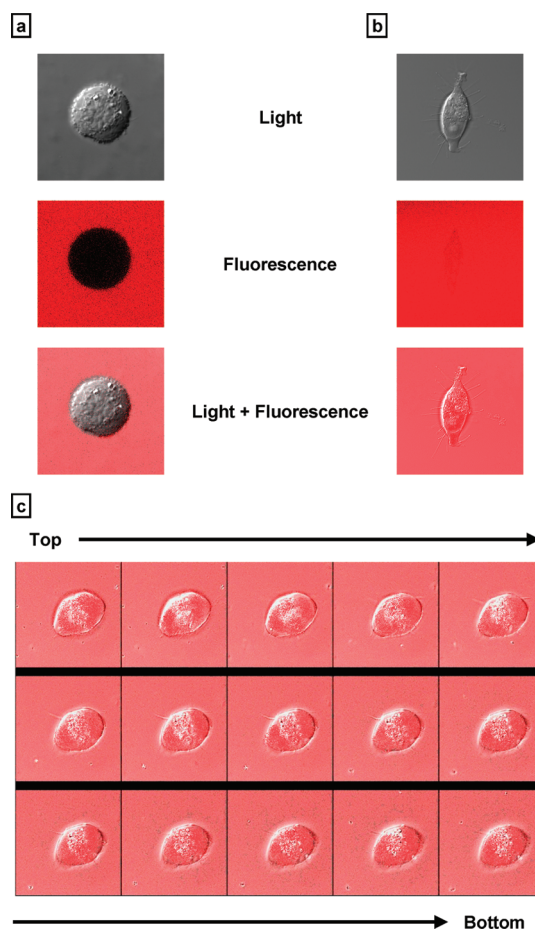


Figure 3. Cellular uptake and localization of naked siRNA and PAMAM-PEG-PLL-siRNA complexes. Representative confocal microscopy images of cancer cells incubated with fluorophore labeled siRNA (siGLO Red, red fluorescence): (a) naked siRNA; (b) PAMAM-PEG-PLL-siRNA; (c) optical section z-series of cells incubated with PAMAM-PEG-PLL-siRNA.

The stability of siRNA in the blood serum was determined by incubating siRNA either naked or complexed with PAMAM-PEG-PLL nanocarrier in the human blood serum (Figure 4). As expected, naked siRNA started to degrade after 1 h of incubation and completely degraded within 12 h. In contrast, complexation of siRNA to PAMAM-PEG-PLL nanocarrier protected siRNA from the nuclease degradation; even 48 h after the incubation of complexed siRNA with human blood serum, siRNA remained nondegraded. Therefore, the proposed complexation of siRNA with PAMAM-PEG-PLL prevents the degradation of siRNA in the plasma.

The gene knock down efficiency of siRNA delivered by poly-L-lysine (PLL), PEG-PLL, PAMAM, PAMAM-PEG, and PAMAM-PEG-PLL nanocarriers with appropriate controls (fresh media, naked specific siRNA, naked nonspecific siRNA with scrambled sequence, and nonspecific siRNA delivered by PAMAM-PEG-PLL nanocarrier) was investigated using quantitative RT-PCR. We selected the BCL2 protein responsible for cellular anti-apoptotic defense as a target for siRNA. The results of these experiments are shown in Figure 5. It was found

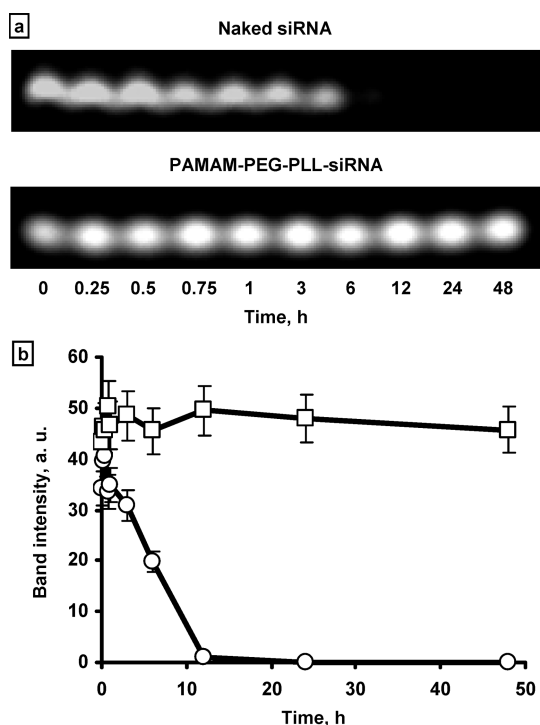


Figure 4. Serum stability of naked and complexed siRNA. (a) Representative images of agarose gel electrophoresis of naked siRNA and PAMAM-PEG-PLL-siRNA complexes. (b) Quantitative analysis of band intensity. Means \pm SD are shown.

that siRNA delivered by PAMAM, PAMAM-PEG, PLL, and PEG-PLL nanocarriers lowered the expression of the targeted gene approximately up to 70–50% from its control value (Figure 5, bars 2 and 3, $P < 0.05$). In contrast, delivery of siRNA by a PAMAM-PEG-PLL triblock nanocarrier led to a significant suppression of the expression of the targeted *BCL2* gene down to 20% from the control value ($P < 0.05$). The decrease in gene expression after incubation with PAMAM-PEG-PLL-siRNA was statistically significant ($P < 0.05$) when compared with either PLL-siRNA or PEG-PLL-siRNA complexes. It should be stressed that naked *BCL2*-specific siRNA, naked nonspecific siRNA, and nonspecific *BCL2* siRNA conjugated with PAMAM-PEG-PLL did not influence the expression of *BCL2* mRNA.

Studies on PLL as a cationic nanocarrier for gene transfection efficiency revealed that PLL alone provides relatively low gene knock down, which is attributed to the lack of tertiary amine groups for the so-called proton sponge effect. It is believed that this effect plays a substantial role in endosomal escape of siRNA inside cells after endocytosis.^{28–30} Nevertheless, PLL in combination with proton sponge ligands such as imidazole or histidine effectively reduced the gene expression; however, the toxicity of PLL dramatically decreased when imidazole or histidines were attached to PLL.^{19,20} In the present investigation, we propose a combination of PLL with a nontoxic PAMAM-NHAc dendrimer that possesses several internal tertiary

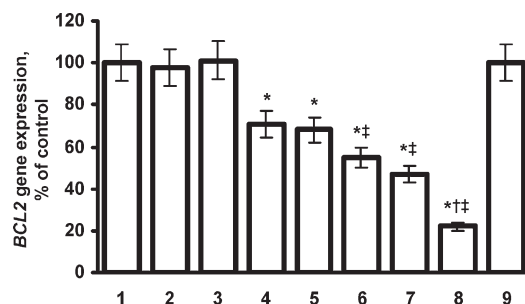


Figure 5. Expression of *BCL2* gene in A2780 human ovarian cancer cells incubated with (1) control (fresh media); (2) naked *BCL2* siRNA; (3) naked nonspecific siRNA; (4) PAMAM-*BCL2* siRNA; (5) PAMAM-PEG-*BCL2* siRNA; (6) PLL-*BCL2* siRNA; (7) PEG-PLL-*BCL2* siRNA; (8) PAMAM-PEG-PLL-*BCL2* siRNA; (9) PAMAM-PEG-PLL-*BCL2* nonspecific siRNA. Means \pm SD are shown. * $P < 0.05$ when compared with control. † $P < 0.05$ when compared with PLL-siRNA. ‡ $P < 0.05$ when compared with PAMAM-siRNA.

amine groups. It is expected that these groups will induce osmotic swelling of the endosome due to endosomal buffering and lead to the rupture of endocytotic vesicles and subsequent release of their payload. Furthermore, polyethylene glycol (PEG) was included in the nanocarrier to enhance siRNA stability against nuclease enzymes during the voyage in the human bloodstream. We also achieved a decrease in cytotoxicity of PLL by attaching a nontoxic PAMAM-NHAc dendrimer and polyethylene glycol.

The ability of PLL, PEG-PLL, and PAMAM-PEG-PLL to form a complex with siRNA was compared using the agarose gel electrophoresis method. All three nanocarriers formed a stable complex at a N/P ratio of 1 and above. The numbers of cationic primary amine groups were calculated based on PLL molecular weight and degree of polymerization (~ 8000 Da and 57, respectively). Each PAMAM-PEG-PLL carrier (calculated $M_w = 27\,650$ Da) contained approximately 56 primary amine groups. Similarly, cationic groups for PEG-PLL (calculated $M_w = 11\,000$ Da, DP 57) and PLL ($M_w = 8000$ Da, DP 57) were calculated as 56 and 57, respectively. As expected, the agarose gel electrophoresis data showed that PAMAM-PEG-PLL was similar to the PEG-PLL and PLL ability to form complexes with siRNA. Dynamic light scattering data revealed an average size around 150 nm of the resulting complexes of the proposed nanocarriers with siRNA. This size of the resulting nanoparticles and possible impact of PLL as a penetration enhancer resulted in the efficient cellular uptake of triblock nanocarrier PAMAM-PEG-PLL-siRNA complexes by human cancer cells.

However, effective uptake of siRNA by cells does not automatically ensure effective silencing of its targeted mRNA. For instance, previously, we have shown that an effective intracellular delivery of siRNA by dendrimers does not guarantee its high gene silencing activity.^{13,14} Down-regulation of a specific gene by siRNA can be controlled by two possible contributing factors: (1) effective cellular internalization of siRNA and (2) endosomal

escape of the payload to perform the task. Some cationic polymers used for siRNA delivery including PLL polymer show an excellent penetration into the cells, while demonstrating a relatively weak gene knock down due to poor endosomal release of the siRNA payload.³¹ The PAMAM dendrimer unit in the triblock of the proposed nanocarrier PAMAM-PEG-PLL provides the required tertiary amines for proton sponge effect and subsequent endosomal release of the siRNA. We would like to note that proton sponge effect is only one possible mechanism of the release of siRNA from the complex. The following mechanisms can potentially be involved in the intracellular release of siRNA. First, siRNA-carrier complex enters the cells by endocytosis in membrane-limited endosomes that eventually fuse with lysosomes. This leads to the sharp decrease in pH disrupting electrostatic interactions between the nucleic acid and carrier and ultimately leading to the siRNA release. Second, lysosomal enzymes and the acidic environment can either degrade or swell polymers, stimulating the release of siRNA from the nanoparticle.³² Third, polymers can themselves possess some membrane disruptive properties. They can swell and burst the endosome through protonation of excess amine groups.³³ The exact mechanisms of intracellular release of siRNA require further more detailed investigation.

Thus, the PAMAM-PEG-PLL nanocarrier fulfills both the requirements of an effective delivery system of improved penetration and delivery of siRNA to the cytoplasm to achieve desired gene knock down. The role of the PAMAM dendrimer was confirmed by comparing the gene silencing efficiency of the BCL2 gene of siRNA complexed with triblock PAMAM-PEG-PLL, PLL, and PEG-PLL nanocarriers. Indeed, the triblock nanocarrier PAMAM-PEG-PLL-siRNA showed maximum suppression of the expression of the targeted

BCL2 gene, while PLL alone or in combination with poly(ethylene glycol) (PLL-PEG) led to a substantially lower decrease in the expression of this gene.

After confirming the role of PLL and PAMAM in the triblock nanocarrier PAMAM-PEG-PLL, we further explored the role of PEG to protect the siRNA during the voyage in the human bloodstream. Nuclease enzyme degradation of siRNA in the blood serum is one of the major obstacles for the *in vivo* therapeutic applications of the siRNA. We and others have reported that PEGylation of siRNA or nanocarriers greatly improved the stability of the siRNA in the human blood serum.^{34–38} Though the exact mechanisms of such stabilization are not clear, one can assume that siRNA is shielded by a linear polymer polyethylene glycol and thus minimizes its exposure to the nuclease enzymes. This assumption is based on the following considerations. Although PEG is a middle block of the nanocarrier, it is also a hydrophilic segment and therefore one can expect a micelle-like geometry of the complex. Though we do not have any evidence, we believe that the triblock nanocarrier on complexation with siRNA forms a micelle wherein the hydrophilic region (PEG) encapsulates the PLL/siRNA complex. As expected, siRNA complexed with the proposed triblock nanocarrier PAMAM-PEG-PLL showed excellent siRNA stability in human blood serum. In fact, complexed siRNA was stable in the human serum more than 48 h, while naked siRNA degraded in less than 6 h.

CONCLUSIONS

A triblock nanocarrier was designed, synthesized, and evaluated for the efficient delivery of siRNA. The multifunctional triblock nanocarrier is synthetically simple to prepare and provides a solution to several obstacles involved in therapeutic applications of siRNA.

METHODS

Materials. Generation four PAMAM-NH₂ dendrimers ($M_w \sim 14\,214$ Da, 64 amine end groups), PLL-HBr ($M_w \sim 12\,000$, degree of polymerization equal to 57), 4-(methylamino)pyridine, and methyl iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO). α,ω -Bis(2-carboxyethyl)polyethylene glycol ($M_w \sim 3000$ Da) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride were obtained from Fluka (Allentown, PA). Spectra/Por dialysis membranes were obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Ethidium bromide (EtBr) solution was purchased from Promega (Madison, WI). The sequence of siRNA targeted to BCL2 mRNA custom synthesized by Ambion (Austin, TX) was 5'-GUG AAG UCA ACA UGC CUG C-dTdT-3' (sense strand) and 5'-GCA GGC AUG UUG ACU UCA C-dTdT-3' (antisense strand). Nonspecific siRNA used as a negative control (sense strand, 5'-CCU CGG GCU GUG CUC UUU U-dTdT-3'; antisense strand, 5'-AAA AGA GCA CAG CCC GAG G-dTdT-3') was received from Dharmacon Inc. (Lafayette, CO). Fluorescent RNA duplex, siRNA labeled with Pierce NuLight DY-547 fluorophores (siGLO Red Transfection Indicator, red

fluorescence), was obtained from Applied Biosystems (Ambion, Inc., Foster City, CA). All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ).

Cell Line. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Fisher Scientific, Fairlawn, NJ). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Synthesis of Surface-Modified PAMAM Dendrimer (PAMAM-NHAc). The surface-modified and partially acetylated PAMAM-NHAc dendrimer was prepared using a previously reported procedure¹³ (Scheme 1). Briefly, triethylamine (0.11 mL, 0.82 mmol) was added to a stirred solution of PAMAM-NH₂ generation four dendrimer (172 mg, 0.012 mmol) dissolved in anhydrous methanol (10 mL) followed by the addition of excess acetic anhydride (0.08 mL, 0.72 mmol). The resulting mixture was stirred at room temperature for 24 h. Methanol was evaporated under reduced pressure and the resulting residue was dissolved in water (2 mL). Further purification by extensive dialysis against

deionized water using dialysis membrane (molecular mass cutoff = 2000 Da) and freeze-drying afforded acetylated PAMAM dendrimer. The degree of acetylation was confirmed by proton nuclear magnetic resonance ($^1\text{H NMR}$).

Synthesis of PAMAM-PEG-COOH Conjugate. α,ω -Bis(2-carboxyethyl)-polyethylene glycol (15 mg, $5\ \mu\text{mol}$, $M_w \sim 3000$ Da) and PAMAM-[(NHAC)₅₈(NH₂)₆] dendrimer (83 mg, $5\ \mu\text{mol}$) were dissolved in the mixture of anhydrous solvents of methylene chloride (5 mL) and dimethyl sulfoxide (5 mL) (Scheme 1). After stirring for 10 min at room temperature, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl) (1 mg, $5.3\ \mu\text{mol}$) and 4-(methylamino)pyridine (DMAP) (0.5 mg) were added to the reaction mixture. The resulting mixture was stirred for an additional 36 h at room temperature, and solvents were removed under reduced pressure. The residue was dissolved in water and purified by extensive dialysis using Spectra/Por dialysis membrane (molecular weight cutoff, MWC = 6000 Da) against deionized water. The conjugate was further purified by passing through a Sephadex G10 column using water as eluent and lyophilized to obtain PAMAM-PEG-COOH as a white solid.

Synthesis of PAMAM-PEG-PLL Conjugate. Triethylamine (0.2 mL) was added to a stirred solution of poly-L-lysine hydrobromide (22 mg, $1.83\ \mu\text{mole}$, $M_w \sim 12\ 000$, degree of polymerization equal to 57) in anhydrous dimethyl sulfoxide (3 mL) (Scheme 1). The reaction mixture was further diluted with anhydrous methylene chloride (5 mL) followed by the addition of PAMAM-PEG-COOH conjugate (22 mg, $1.14\ \mu\text{mol}$) and stirred at room temperature for 15 min. EDC·HCl (1.5 mg, $7.8\ \mu\text{mol}$) and DMAP (0.5 mg) were added to the reaction mixture. The resulting solution was stirred for an additional 36 h at room temperature. The side product carbodiimide urea was filtered off, and solvents were removed under reduced pressure. The residue was dissolved in water and purified by extensive dialysis using Spectra/Por dialysis membrane (MWC = 25 000 Da) against deionized water. The conjugate was further purified by passing through a Sephadex G10 column using water as eluent and lyophilized to obtain PAMAM-PEG-PLL as a hygroscopic white solid.

Synthesis of PEG-PLL Conjugate. NHS-PEG-OMe (15.6 mg, $3.1\ \mu\text{mol}$, $M_w \sim 5000$) in phosphate buffer (pH 8.4) was added to a stirred solution of poly-L-lysine hydrobromide (22 mg, $1.83\ \mu\text{mole}$, $M_w \sim 12\ 000$, degree of polymerization equal to 57) in phosphate buffer (pH 8.4). The resulting solution was stirred for 6 h at room temperature. The resulting reaction mixture was then dialyzed against 1 N HCl for 12 h and subsequently extensively dialyzed against deionized water using dialysis membrane Spectra/Por (MWC = 8000 Da). Further purification by passing through a Sephadex G10 column using water as eluent and freeze-drying afforded the PEG-PLL conjugate.

Synthesis of PAMAM-PEG-PLL-Cy5.5. Cy5.5 mono-NHS ester (1.5 mg, $1.32\ \mu\text{mol}$) dissolved in anhydrous dimethyl sulfoxide (1 mL) was added to a stirred solution of PAMAM-PEG-PLL (9 mg, $0.32\ \mu\text{mol}$, $M_w \sim 28\ 000$) in 0.1 mM NaHCO₃ (1 mL). The resulting mixture was stirred in the dark at room temperature for 6 h. Extensive dialysis using Spectra/Por dialysis membrane (MWC = 25 000) against deionized water was carried out to remove unreacted Cy5.5. Additionally, the conjugate was purified by passing through a Sephadex column. The concentration of Cy5.5 dye attached to the PAMAM-PEG-PLL nanocarrier was estimated by measuring its fluorescence (excitation 675 nm, emission 694 nm) using Cy5.5 NHS ester as standard.

Proton Nuclear Magnetic Resonance Spectroscopy ($^1\text{H NMR}$). $^1\text{H NMR}$ was performed on a Varian VNMRs 400 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA). The chemical shift was expressed as parts per million (ppm), and a solvent peak was used for reference (D₂O, 4.8 ppm). The following abbreviations are used in the Results section to identify multiplicities of spectra peaks: s, singlet; m, multiplet; br, broad.

In Vitro Cytotoxicity. A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cytotoxicity of the following nanocarriers PEG, PLL, PEG-PLL, PAMAM-NH₂, PAMAM-OH, PAMAM-NHAC, and PAMAM-PEG-PLL as previously described.^{39,40} To measure cytotoxicity, cells were separately incubated in a microtiter plate with different concentrations of PLL, PEG-PLL, and PAMAM-PEG-PLL. Control

cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. On the basis of these measurements, cellular viability was calculated for each nanocarrier concentration. A decrease in the cellular viability indicated an increase in the toxicity.

Characterization of siRNA Complexation with Nanocarriers. The complexes of cationic nanocarriers (PLL, PEG-PLL, and PAMAM-PEG-PLL) and siRNA were prepared in water at N/P (amine/phosphate) charge ratios ranging from 0 to 1.5 and incubated at room temperature for 30 min. The charge ratio was calculated by relating the number of cationic primary amine groups on the nanocarrier with the number of negatively charged phosphate groups of siRNA. Dendrimer-free siRNA was used as the control. Double-stranded RNA ladder (New England Biolabs, Ipswich, MA) with the smallest base pairs at 21 was used as a size reference. The samples were further diluted with DPBS buffer and electrophoresed in 4% agarose gel at 100 V for 50 min in Tris-borate-EDTA buffer containing ethidium bromide. The siRNA bands on the gel were visualized under ultraviolet light and photographed. Complex formation was also quantified by measuring fluorescence of ethidium bromide in the sample at 530 nm excitation and 590 nm emission wavelengths.⁴¹ The fluorescence intensity at N/P charge ratio equal to 0 was set to 100%.

Dynamic Light Scattering (DLS) Analysis and Zeta-Potential. PAMAM-PEG-PLL-siRNA complex was prepared by mixing PAMAM-PEG-PLL and siRNA in water at a N/P ratio equal to 3. The resulting complex was incubated for 30 min, and the size was determined using the DynePro-MS800 dynamic light scattering/molecular sizing instrument with an argon laser wavelength $\lambda = 830$ nm, a detector angle 90°, and typical sample volume of 20 μL . Each light scattering experiment consisted of 20 or more independent readings, 10 s in duration each. Data analysis was conducted using DynaPro Instrument Control Software for molecular Research DYNAMICS (version 5.26.60). The obtained DLS data represent the average of three runs. Zeta-potential was measured on PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY). Samples were taken as is, and their volume was 1.5 mL. All measurements were carried out at room temperature. Each parameter was measured five times, and average values were calculated.

Cellular Internalization. Cellular uptake and intracellular localization of siRNA was investigated using a confocal microscopy. In this experiment, living cancer cells were incubated with fluorophore-labeled naked siRNA (siGLO Red, red fluorescence) and siRNA complexed with cationic nanocarrier PAMAM-PEG-PLL (N/P = 3). Cellular uptake substances were monitored in living cells placed in a chamber at 37 °C within 1 h. Cellular localization of siRNA was examined on fixed and washed cells after the incubation for 24 h with the substances. Fluorescence and its distribution within the cell were examined using a confocal microscope.

Gene Expression. Reverse transcription polymerase chain reaction (RT-PCR) was used for the analysis of gene expression as described previously.⁴⁰ The cationic nanocarrier (PLL, PLL-PEG-OMe, and PAMAM-PEG-PLL)-siRNA (for *BCL2* gene) complexes were added to the cells with the final concentration of siRNA equal to 1 μM . After 24 h, total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Piscataway, NJ) with 2 μg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences, Piscataway, NJ). After synthesis, the reaction mixture was immediately subjected to polymerase chain reaction (PCR), which was carried out using GenAmp PCR System 2400 (Perkin-Elmer, Shelton, CT). β_2 -microglobulin (β_2 -m) was used as an internal standard. The following pairs of primers were used: *BCL2*: 5'-GGA TTG TGG CCT TCT TTG AG-3' (sense), 5'-CCA AAC TGA GCA GAG TCT TC-3' (antisense); β_2 -m (internal standard)-ACC CCC ACT GAA AAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR regimen was as follows: 94 °C for 5 min; 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 41 cycles; and 60 °C for 10 min. PCR products were separated in 4% NuSieve 3:1 Reliant agarose gels (Lonza, Basel, Switzerland) in 1 \times Tris-borate EDTA buffer [0.089 mol/L Tris-borate, 0.002 mol/L

EDTA (pH 8.3); Research Organics Inc., Cleveland, OH] by submarine electrophoresis. The gels were stained with EtBr and digitally photographed.

Serum Stability of siRNA and PAMAM-PEG-PLL-siRNA Complex. Serum stabilities of naked siRNA and siRNA complexed with PAMAM-PEG-PLL nanocarrier were investigated by incubating siRNA or PAMAM-PEG-PLL-siRNA complex in 50% human serum at 37 °C. Ten samples were prepared by mixing siRNA (30 nmol) in water with PAMAM-PEG-PLL (74.8 nmol, N/P = 3) solution in water and separately incubated for 30 min at room temperature. In the case of naked siRNA, equal volume of RNAase-free water was used instead of nanocarrier. To each of these samples was added 50% human plasma (final siRNA concentration was 1.43 nM) and incubated at 37 °C. Samples were removed at indicated time intervals (0, 0.25, 0.5, 0.75, 1, 3, 6, 12, 24, and 50 h) and analyzed using a gel electrophoresis (4% agarose gel at 100 V for 50 min) in Tris-borate-EDTA buffer containing EtBr. The siRNA bands on the gel were visualized under ultraviolet light. PAMAM-PEG-PLL-siRNA samples were pretreated with polymethacrylic acid to release free siRNA from cationic nanocarriers. Then, 100 μ L of polymethacrylic acid solution (4 μ M) was added to the complexes (triblock nanocarrier/siRNA = 3) and incubated at 37 °C with 50% human plasma. The released siRNA was then analyzed by gel electrophoresis.

Statistical Analysis. Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value \pm standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student's *t* test and considered *P* values of less than 0.05 as statistically significant.

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