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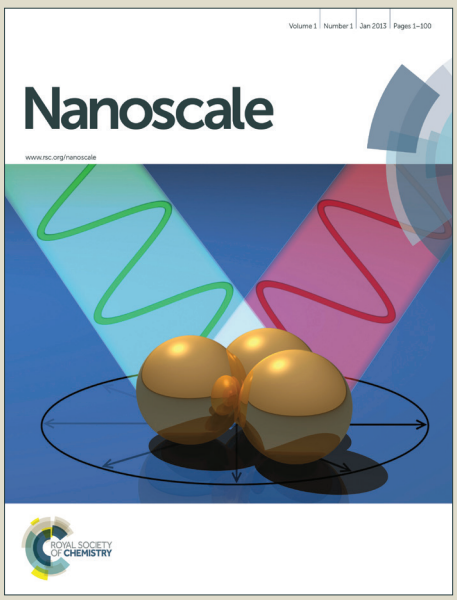

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Promoting siRNA delivery via enhanced cellular uptake using an arginine-decorated amphiphilic dendrimer

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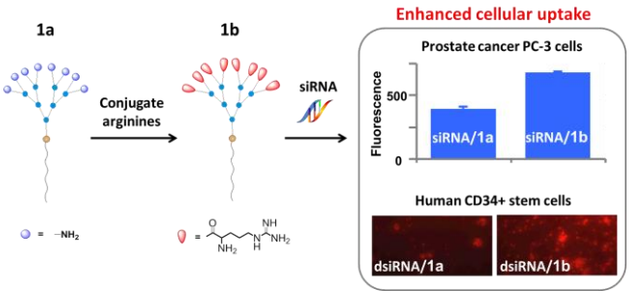
An arginine-decorated amphiphilic dendrimer is able to promote enhanced cellular uptake of siRNA, leading to considerably improved siRNA delivery and gene silencing.



Abstract

RNA interference (RNAi) with small interfering RNA (siRNA) is expected to offer an attractive means to specifically and efficiently silence disease-associated genes for treating various diseases provided that safe and efficient delivery systems are available. In this study, we have established an arginine-decorated amphiphilic dendrimer composed of a hydrophobic alkyl chain and a hydrophilic PAMAM dendron bearing arginine terminals as nonviral vector for siRNA delivery. Indeed, this dendrimer proved to be very effective at delivering siRNAs in human prostate cancer PC-3 cells and in human hematopoietic CD34+ stem cells, leading to improved gene silencing compared to the corresponding nonarginine decorated dendrimer. Further investigation confirmed that this dendrimer was granted with the capacity to form stable nanoparticles with siRNA and significantly enhance cellular uptake of siRNA. In addition, this dendrimer revealed no discernible cytotoxicity. All these findings demonstrate that decoration of the dendrimer surface with arginine residues is indeed a useful strategy to improve the delivery ability of dendrimers.

Introduction

The application of nanotechnology for drug delivery is widely expected to bring promise and create novel therapeutics. Particularly exciting is the recent effort to apply nanotechnology to engineer nanovectors for small interfering RNA molecules (siRNAs) delivery, giving birth to entirely novel siRNA therapeutics. RNA interference (RNAi) makes use of siRNA, which can break down the target mRNA in a sequence-specific manner.[1] The promise that siRNA can efficiently and specifically down-regulate disease-associated or even "undruggable" genes offers the compelling advantage to treat various diseases, creating a new era of pharmaceutical science which have the potential to revolutionize traditional medicine.[2-4]

However, as a nucleic acid component, its intrinsic physicochemical properties make siRNA extremely poor as an active pharmaceutical ingredient and pose serious obstacles for clinical translation of RNAi. Generally, an unprotected, naked siRNA will be rapidly degraded by nucleases and/or esterases circulating in the biological fluids. In addition, siRNA molecules are highly negatively charged and hydrophilic, and hence do not readily cross cell membranes. Therefore, a safe and robust delivery system which can protect siRNA from degradation during the extra- and intra-cellular delivery and bring siRNA to the site of interest is an essential prerequisite of success clinical development of RNAi.

In the past decades, a large number of nanovectors, such as lipids, natural macromolecules (e.g. cyclodextrin, chitosan, polysaccharide), organic polymers (e.g. polyethylenimine, dendrimers) or inorganic materials (e.g. carbon nanotubes, quantum

dots, gold nanoparticles), have been established for siRNA delivery.[5-9] Among these, cationic dendrimers, a family of synthetic polymers,[10] represent a particularly promising siRNA delivery platform[11-13] by virtue of their precisely constructed structure, intriguing multivalency and high cargo payload confined within a nanosized volume. We have recently reported a small amphiphilic poly(amidoamine) (PAMAM) dendrimer (**1a** in Figure 1) featuring a hydrophobic long alkyl chain and a low generation hydrophilic PAMAM dendron for functional siRNA delivery.[14] This dendrimer is able to harness the advantageous delivery features of both lipid and polymer vectors, successfully delivering siRNA and producing potent gene silencing and anticancer activity in prostate cancer models in vitro and in vivo.[14]

In order to further improve the delivery efficiency of this amphiphilic dendrimer *via* increased cellular uptake of siRNA, we would like to conjugate this amphiphilic dendrimer with arginine entities (**1b** in Figure 1A). Our rationale for such initiation was motivated by several considerations. First of all, polyarginine peptides are well known as one of the widely used cell-penetration peptides for intracellular delivery system,[15, 16] and various delivery vehicles containing arginine-rich motifs have been reported for their improved cellular uptake to transport various therapeutics, such as small molecular drugs, proteins, nucleic acids, etc.[15, 17-23] Secondly, the arginine residue harbors two positively charged functional groups at physiological pH 7.4, namely, the primary amine functionality and the guanidinium moiety, both are able to interact favorably with the negatively charged cell membrane-associated proteoglycans and the phosphate groups in lipid components, hence facilitating membrane penetration.[24-26] Finally, it has

been demonstrated that certain number of arginine residues and length of the arginine peptide are required for the optimal cellular uptake.[15] On the basis of the multivalent property of dendrimers, we inferred that the dendrimers bearing single arginine unit terminals could be considered as arginine-rich nanovectors. Indeed, our recent work with a triethanolamine (TEA) core poly(amidoamine) (PAMAM) dendrimer harboring single arginine units at its terminals showed significantly increased cellular uptake of siRNA, hence enhancing gene silencing efficiency in comparison with the corresponding nonarginine bearing dendrimer counterpart.[27]

Based on all these rationales as well as our promising results obtained with the amphiphilic dendrimer **1a**[14] and with the arginine-conjugated TEA-core PAMAM dendrimer,[27] we expected that the arginine-terminated amphiphilic dendrimer **1b** could conserve the cell penetration advantage of arginine-rich motifs, as well as the beneficial features of the amphiphilic dendrimers for siRNA delivery.[14, 28, 29] We present below the results that this amphiphilic dendrimer **1b** enhanced siRNA delivery in prostate cancer cells as well as in human stem cells, and demonstrate that the improvement in delivery efficiency can be indeed attributed to the advantageous cell penetration ability imparted by the arginine terminals within the amphiphilic dendrimer **1b**.

Experimental

Materials.

The amine-terminated amphiphilic dendrimer **1a** was synthesized according to the previously reported method.[14] All chemicals were purchased from Acros Organics, Sigma Aldrich or Alfa Aesar (France). Methyl acrylate and ethylenediamine were

distilled before use. All the other reagents and solvents were used without any further purification. ^{1}H NMR spectra were recorded at 600 MHz and ^{13}C NMR spectra recorded at 75 MHz on Varian Mercury-VX600 and Varian Mercury-VX300 spectrometers, respectively. Coupling constants (*J*) are reported in Hertz, and chemical shifts are reported in parts per million (ppm). ESI mass spectra were determined using Finnigan LCQ Advantage mass spectrometer. IR spectra were recorded with a Nicolet 380 spectrophotometer.

The sequence of heat shock protein 27 (Hsp27) siRNA used corresponds to the human Hsp27 site (5'-GCUGCAAAAUCCGAUGAGACdTdT-3') (Thermo Fisher Scientific, Illkrich, France). A scrambled siRNA duplex (Thermo Fisher Scientific) (5'-AUCAAACUGUUGUCAGCGCUGdTdT-3') and Dy647 labelled Hsp27 were purchased from Thermo Fisher Scientific. Cy3-labeled *Tat/Rev* Site I dsiRNA (27 mer, Sense: 5'-GCG GAG ACA GCG ACG AAG AGC UCA UCA-3'; antisense: 5'-UGA UGA GCU CUU CGU CGC UGU CUC CGC dTdT-3'), Anti-CD4 dsiRNA (Sense: 5'-GAU CAA GAG ACU CCU CAG UGA GAA G-3'; antisense: 5'-CUU CUC ACU GAG GAG UCU CUU GAU CUG-3' (2'-OMe modified U was underlined), and irrelative dsiRNA (NC1 dsiRNA) were purchased from Integrated DNA Technologies (Coralville, IA). Oligofectamine and RNAiMax were purchased from Life Technologies (Paisley, United Kingdom).

All other reagents and solvents of analytical grade were used without any further purification from commercial sources.

Synthesis of the arginine-terminated amphiphilic dendrimers 1b

To a solution of 4.0 eq (equivalent to one primary amine terminal of the amine-terminated dendrimer **1a**) Fmoc-Arg(pbf)-OH, 4.0 eq 1-hydroxybenzotriazole (HOBt), 4.0 eq *O*-(benzotriazol-1-yl)-*N, N, N′, N′*-tetramethyluronium hexafluorophosphate (HBTU) and 8.0 eq *N*, *N*-diisopropylethylamine (DIPEA) in 5.0 mL DMF stirring at 25 ^{o}C under argon, was added a solution of 50 mg of the amine-terminated dendrimer **1a** in 5.0 mL DMF.[27] The resulting solution was stirred at 25^{o}C under argon until completion of the reaction as indicated by TLC analysis. DMF was removed under reduced pressure, and the residue was purified by precipitation with CH_3OH/Et_2O (1.0 mL / 20 mL) at 4 ^{o}C for 12 h (three times). The residual precipitate was collected, dried by rotavap and then dissolved in 3.0 mL DMF and mixed with a solution of 3 mL 30% piperidine in DMF (v/v). The mixture was stirred at 25 ^{o}C under argon for 2 h, and DMF was then removed under reduced pressure. The residue was purified by precipitation with CH_3OH/Et_2O (1.0 mL / 20 mL) at 4^{o}C for 12 h (three times). A solution of 7.6 mL trifluoroacetic acid (TFA), 2.0 mL triisopropylsilane (TIS) and 2.0 mL water was added to the residual precipitate and stirred 25 ^{o}C under argon for 1 h. The crude product was dissolved in water (1-2 mL) and subjected to dialysis in water (800-1000 mL) (dialysis protocol: at the beginning, change water every 1 h for three times, then change water every 2-3 h for another three times). Then the product was lyophilized to offer the corresponding product **1b** with a yield of 90%, which was analyzed using IR, ^{1}H- and ^{13}C-NMR. The resulting degree of modification with arginine was almost quantitative according to the molar ratio of Arg/PAMAM assessed by quantifying the corresponding NMR peaks at 3.8 ppm and 2.4 ppm for arginine

residue and PAMAM dendron, respectively.

^{1}H NMR (600 MHz, D_2O): δ 7.87 (s, 1H, CH), 4.26 (t, 2H, *J* = 6.3 Hz, CH_2), 3.76-3.81 (m, 10H, CH_2), 3.11-3.28 (m, 28H, CH_2), 3.05 (t, 16H, *J* = 6.9 Hz, CH_2), 2.91-2.96 (m, 28H, CH_2), 2.66-2.80 (m, 28H, CH_2), 2.37-2.43 (m, 28H, CH_2), 1.71-1.79 (m, 18H, CH_2), 1.46-1.48 (m, 16H, CH_2), 1.00-1.05 (m, 30H, CH_2), 0.65 (t, 3H, *J* = 6.6 Hz, CH_3); ^{13}C NMR (75 MHz, D_2O): δ 173.8, 173.4, 170.2, 157.5, 156.9, 117.4, 115.5, 53.1, 53.0, 51.8, 49.4, 40.4, 39.2, 38.8, 35.6, 32.3, 29.1, 28.2, 23.7; IR (cm^{-1}): υ 1630.3; MS: calcd. for $C_{137}H_{278}N_{64}O_{22}^{+}$ $[M+H]^+$ 3197.3, found 3198.4; HRMS: calcd. for $C_{139}H_{278}N_{84}O_{22}Na^{+}$ $[M+Na]^+$ 3220.2520, found 3220.2493.

Agarose gel analysis of siRNA/dendrimer complexes.

Dendrimers were diluted to an appropriate concentration in 50 mM Tris-HCl buffer (pH 7.4), with all solutions stored at 4 ℃. The siRNA was diluted with H_2O. Both solutions were mixed at various N/P ratios and incubated at 37 ℃ for 30 min. The final concentration of siRNA was adjusted to 25 ng/mL (100 ng/well). siRNA/dendrimer complexes were analyzed by electrophoretic mobility-shift assays in 2% agarose gel in 0.5×TBE buffer. The siRNA bands were stained by syber green (Life Technologies SAS, Saint Aubin, France) then detected by Fujifilm LAS-3000 Imager (FUJIFILM, Bois d'Arcy, France).

Dynamic light scattering (DLS) analysis

The siRNA solution was mixed with the indicated amount of freshly prepared dendrimer solution at an N/P ratio of 10. The final concentration of the siRNA was 1 μM. After incubation at 25 ℃ for 30 min, size distribution and zeta potential

measurements were performed using Zetasizer Nano-ZS (Malvern Ltd., Malvern, Orsay, France) with a He-Ne ion laser of 633 nm.

Cell culture.

Human prostate cancer PC-3 cells were purchased from the American Type Culture Collection (Molsheim, France). PC-3 cells were maintained in DMEM (Invitrogen Ltd., Paisley, United Kingdom), supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37 ℃ in a 5% CO_2 humidified atmosphere.

Human hematopoietic CD34+ stem cells: Cord blood samples were obtained from StemCyte, Inc (Covina, CA). For transfection of hematopoietic stem cells, CD34+ cells were enriched from umbilical cord blood by anti-CD34 antibody-coupled magnetic beads (Miltenyi Biotech, Aubum, CA). Freshly isolated CD34+ cells were cultured in active medium for 3 days. Forty-eight hours prior to transfection, the CD34+ cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1×PenStrep and 100 U/mL interleukin-2. Cells should be cultured on the Non-Tissue Culture Treated plates (Becton Dickinson Labware).

Cellular uptake studies.

Flow Cytometry.

Briefly, PC-3 cells were plated in 3.5 cm dishes, and treated with Dy647 labeled Hsp27 siRNA/**1b**, using no treatment and Dy647 labeled Hsp27 siRNA/**1a** as controls. The cells were trypsinized after treatment and washed once with cold PBS. The fluorescent intensity of cells was analyzed by using flow cytometer (Beckman Coulter Epics Elite, Beckman Coulter, Inc., Marseille, France). Each assay was performed in triplicate.

Fluorescent Microscopy analysis.

2×10^5 suspension CD34+ cells per well were seeded in 48-well tissue culture plates in 150 μL fresh complete medium containing 10% FBS. Cells were transfected with 50 nM Cy3-labeled *Tat/Rev* Site I dsiRNA (27 mer) using dendrimers as described below. The images were collected after transfection for 24 or 48 h using a Nikon Eclipse TE2000-S fluorescent microscopy system at 20X magnification.

Transfection experiments.

Human prostate cancer PC-3 cells.

One day before transfection, 1.5×10^5 cells were seeded in 6 cm dishes in 4 mL of fresh complete medium containing 10% FBS. Before transfection, a solution of the siRNA/dendrimer, and siRNA/oligofectamine complex was prepared accordingly. The desired amount of siRNA and dendrimers or oligofectamine was diluted in 200 μL of Opti-MEM transfection medium. The solutions were mixed with a vortex for 10 sec and then equilibrated 10 min at room temperature. The dendrimers or oligofectamine was added to the siRNA solution, homogenized for 10 sec with a vortex and equilibrated 30 min at room temperature. Then 1.6 mL of serum-free medium was added into the complex solution and the final volume brought to 2 mL. Before addition of the transfection complexes, the complete medium with FBS was removed and cells were washed with PBS once. Then, 2 mL of complex solution was added and incubated at 37 ℃ in the absence of 10% FBS. After 8 h of incubation, the transfection mixture was replaced with the complete medium containing 10% FBS, and maintained under normal growth conditions for further incubation of 72 h.

Human hematopoietic CD34+ *stem cells*

4×10^5 CD34+ cells per well were seeded in 24-well tissue culture plates in 300 μL fresh complete medium. Before transfection, complexes of dsiRNA/dendrimer reagents were prepared. The desired amount of dsiRNA and dendrimer reagent was diluted in 50 μL of serum-free medium (Opti-MED), respectively. The dendrimer solution was mixed gently and incubated for 10 min at room temperature. After the 10-minute incubation, the diluted dsiRNA and the dendrimer reagent were mixed gently and incubated for 30 minutes at room temperature. The 100 μL of dsiRNA/dendrimer complex was added to each well containing cells and medium and mixed gently by rocking the plate back and forth. The cells were incubated at 37℃ in a CO_2 incubator for 24~48 h for further assays. A commercial transfection reagent RNAiMax (Life Technologies) was used as control according to the manufacturer instructions.

Total RNA isolation and cDNA synthesis.

RNA-Stat60 was used to extract total RNA according to the manufacturer instruction (Tel-Test). The concentration and integrity of total RNA were measured by nano-drop spectroscopy. Residual DNA was digested using the DNA-free kit following manufacturer instructions (Ambion). cDNA was produced using 2 μg of total RNA, Moloney murine leukemia virus reverse transcriptase and random primers in a 15 μL reaction according to the manufacturer instructions (Invitrogen). GAPDH expression was used for normalization of the qPCR data.

qRT-PCR analysis (determination of gene silencing).

For CD4 expression, cells were transfected with 50 nM of 27-mer anti-CD4 siRNA

using dendrimers as described above. 48 hours post-transfection, the total RNA was isolated with STAT-60 (TEL-TEST "B", Friendswood, TX). Expression of the target genes was analyzed by quantitative Real time-PCR using 2× iQ SyberGreen Mastermix (BIO-RAD) and specific primer sets at a final concentration of 400 nM.

Primers were as follows: CD4 forward primer: 5' - GCT GGA ATC CAA CAT CAA GG -3'; CD4 reverse primer: 5'- CTT CTG AAA CCG GTG AGG AC -3'; GAPDH forward primer: 5'- CAT TGA CCT CAA CTA CAT G-3'; GAPDH reverse primer: 5'- TCT CCA TGG TGG TGA AGA C-3' GAPDH expression was used for normalization of the qPCR data.

Western blot analysis (determination of gene silencing).

Samples containing equal amounts of protein (15 μg) from lysates of cultured PC-3 cells were analyzed by Western blot analysis as described previously with 1:5000-diluted anti-human Hsp27 rabbit polyclonal antibody (Enzo Life Sciences, Villeurbanne, France) or 1:2000-diluted anti-human vinculin mouse monoclonal antibody (Sigma Aldrich). Filters were then incubated for 1 h at room temperature with 1:5000-diluted horseradish peroxidase conjugate anti-rabbit or mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Nanterre, France). Specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (GE Healthcare Life Sciences, Velizy-Villacoublay, France).

MTT assay.

The metabolite toxicity of Hsp27 siRNA plus **1b** on PC-3 cells was assessed using the MTT (Sigma Aldrich, France). Briefly, cells were seeded in each well of 12-well

microtiter plates and allowed to attach overnight. Cells were then treated once with dendrimer **1b** and scramble siRNA/**1b** in the absence of 10% FBS. Every 1, 3 and 24 h, MTT solution (5 mg/mL in PBS) was added to each well and incubated for another 2-4 h. And then the suspension liquid was removed and cells were resuspended in DMSO. The optical density (OD) of these DMSO solutions was read at 540 nm. The difference of OD values between treated and non-treated cells reflects the viability of cells after treatments and thus stands for the metabolite toxicity. Each assay was performed in triplicate.

MTS assay.

1×10^5 CD34+ cells per well were seed in 96-well plates in 75 μL fresh complete medium. Before transfection, complexes of dsiRNA/dendrimer reagents were prepared as described above. 25 μL of dsiRNA/dendrimer complex was added to each well containing cells and medium and mixed gently by rocking the plate back and forth. The cells were incubated at 37℃ in a CO_2 incubator for 2 days for the MTS cytotoxicity assay. CellTiter 96® Non-Radioactive Cell Proliferation (Promega) assays were performed according to manufacturer protocol. 20 μL MTS solution was added to each well and then incubated with cells for 1.5 h at 37℃. The absorbance was measured with a Wallack plate reader using the Kamings protocol at 490 nm. Experiments were performed in triplicate. Cell viability was expressed as the ratio of absorbance obtained from transfected cells to non-transfected cells.

Statistical analysis

Statistical analysis was performed by Student's test (t-test) (Microsoft Excel 2003). $p\leqslant$

0.05 was considered significant (*); p≤0.01 (**); p≤0.001 (***).

Results and discussion

Synthesis. The synthetic strategy of the arginine-decorated amphiphilic dendrimer **1b** is illustrated in Figure 2A, which began with the amine-terminated amphiphilic dendrimer **1a** [14] and proceeded by conjugation with the arginine building units. Precisely, the amine-terminated amphiphilic dendrimer **1a** was first coupled with the protected arginine precursor, Fmoc-Arg(pbf)-OH, in the presence of HOBt/HBTU and DIPEA in DMF. Subsequent removal of the Fmoc groups and deprotection of the pbf groups offered the desired arginine-decorated amphiphilic dendrimer **1b** with an excellent yield of 90%.

The successful conjugation of arginine residues to the amine-terminated amphiphilic dendrimer **1a** was confirmed by NMR analysis. For example, the peaks at 1.5 ppm, 1.7 ppm, 3.1 ppm, and 3.8 ppm in the ^{1}H NMR spectrum of the arginine-terminated amphiphilic dendrimer **1b** belong to the characteristic NMR signals of the arginine components, which are distinct from those of the corresponding amine-terminated amphiphilic dendrimer **1a** (Figure 2B), highlighting the successful appendage of arginine residues. Similarly, the characteristic ^{13}C NMR signals relating to the arginine residues were found at 40 ppm, 157 ppm, and 176 ppm in the ^{13}C NMR spectrum of **1b** (Figure 2C), further validating the successful attachment of arginine residues onto the dendrimer surface.

Dendrimer 1b formed nanoparticles with siRNA and protected siRNA from

degradation. Packing siRNA into stable and nanosized complexes by the nonviral vector is of paramount importance for effective delivery. We therefore first examined the complex formation between siRNA and the amphiphilic dendrimer **1b** using dynamic light scattering (DLS) analysis, a method able to determine the size and surface charge of the nanoscale complexes in solution. DLS results revealed that the amphiphilic dendrimer **1b** and siRNA formed uniform nanoparticles with an average size of 50 nm (Figure 3A) and a zeta potential of 22 mV. These results are similar to those observations with **1a**.[14]

We next inspected the complex formation between siRNA and **1b** using gel electrophoresis, a widely used technique for assessing complex formation with nucleic acid. siRNAs migrate towards cathode during gel electrophoresis because of their negative charge, whereas dendrimer binding to siRNA retards siRNA migration in the gel, thereby the degree of complex formation can be inferred. Compared to naked siRNA control, siRNA formed complexes with the dendrimer **1b** showed a significant retardation on migration at an N/P ratio of 5 or above (Figure 3B), indicating stable complex formation of siRNA with **1b**. It is noted that the amine-terminated dendrimer **1a** could completely retard siRNA migration at a lower N/P ratio of 2.5,[14] implying that **1a** exhibited somewhat stronger binding towards siRNA than **1b.** This might be ascribed to the planar and rigid guanidiniums at the distal ends of the arginine terminals of **1b,** making the binding of **1b** towards siRNA less efficient than that of **1a**.

Nevertheless, the siRNA/**1b** complexes are stable enough to effectively protect siRNA against enzymatic degradation. As shown in Figure 3C, naked siRNA was

completely digested by RNase within 5 minutes, whereas siRNA complexed with dendrimer **1b** remained intact even after 60 minutes incubation with the enzyme. This result demonstrates that siRNA was well protected from enzymatic degradation, which is also an additional evidence for the formation of stable siRNA/**1b** complexes. Collectively, all these results affirmed that the arginine-decorated dendrimer **1b** is able to pack siRNA to form stable nanoparticles and to protect siRNA from degradation.

Dendrimer 1b mediated superior siRNA delivery. The so-constructed siRNA/**1b** nanoparticles were indeed effective to deliver siRNA and produce potent gene silencing. Figure 4A displayed the resulting gene silencing of heat shock protein 27 (Hsp27) in the human castration-resistant prostate cancer PC-3 cells following the treatment with a siRNA targeting Hsp27 delivered by **1b**. Hsp27, a ubiquitous small heat shock protein, is an essential player in the development of cancer and drug resistance, and has been recently considered as a novel target for treating drug-resistant prostate and other cancers.[30, 31] Compared to the amine-terminated dendrimer **1a**, the arginine-decorated dendrimer **1b** showed considerably increased inhibition of Hsp27 protein (Figure 4A) with activity comparable to the commercial transfection vector oligofectamine (oligo). Such improved gene silencing with **1b** could be ascribed to the presence of the arginine terminals, which is the sole difference between **1a** and **1b**. Additionally, no discernible cytotoxicity was observed with either **1b** alone or the scramble siRNA/**1b** (Figure 4B).

Most excitingly, **1b** was able to deliver a dicer substrate siRNA (denoted as dsiRNA)[32,33] targeting CD4, the primary receptor for HIV-1, and mediate successful gene silencing in human hematopoietic CD34+ stem cells, a particularly challenging

cell type for nucleic acid delivery. Dendrimer **1b** delivered anti-CD4 dsiRNA resulted in ~ 60% decrease in CD4 mRNA levels (Figure 2C), without any notable cytotoxicity (Figure 2D). In contrast, no inhibition was observed with dendrimer alone or a dendrimer delivered irrelative dsiRNA. The commercial transfection reagent RNAiMax was ineffective for delivery to CD34+ cells (data not shown). Taken together, these results demonstrate that the arginine-decorated amphiphilic dendrimer **1b** was definitely bestowed superior capacity for siRNA delivery compared to its nonarginine dendrimer counterpart **1a**.

Enhanced cellular uptake of siRNA via arginine conjugated dendrimer. To understand how the arginine-terminated dendrimer **1b** exhibits such promoted gene silencing efficiency than the parent amine-terminated dendrimer counterpart **1a**, we inspect the cellular uptake by quantifying the internalization of the siRNA/**1b** complexes using flow cytometry with the far-red fluorochrome Dy647 labeled siRNA. Significantly stronger fluorescent intensity was observed in the cells treated with siRNA/**1b** than those with siRNA/**1a** (Figure 5A), demonstrating the improved cellular uptake ability of siRNA/**1b** complexes compared with their counterpart siRNA/**1a**.

Likewise, the arginine-decorated dendrimer **1b** was able to exercise efficient internalization of siRNA in human hematopoietic CD34+ stem cells. As shown in Figure 5B, whereas no considerable cellular uptake was observed with the Cy3-labeled dsiRNA alone, significant red fluorescent signals were revealed following the treatment of Cy3-labeled dsiRNA/**1b** complexes, entailing effective cell uptake. Importantly, the fluorescent signals were stronger than those treated with Cy3-labeled siRNA delivered

by **1a**. Altogether, these results speak for the predominant cellular uptake of siRNA delivered by **1b**.

The observed advantageous cellular uptake mediated by **1b** in comparison to **1a** can be reasonably attributed to the arginine terminals, the sole structural difference between **1b** and **1a**. Importantly, the positively charged guanidinium terminals, which, via bidentate hydrogen bonding, are able to form strong and important hydrogen bond networks with the negatively charged cell membrane constituents such as carboxylates, phosphates and sulfates. The so-resulting H-bond formation would promote their association with the cell membrane, hence enhancing cell membrane penetration and internalization.[15, 34] Therefore, appending arginine residues on the dendrimer surface effectively improves the cellular uptake ability of siRNA, and the arginine-terminated amphiphilic dendrimer **1b** is thereby able to exhibit enhanced ability to transport siRNA and mediate more efficient gene silencing than the nonarginine bearing dendrimer **1a**.

CONCLUSIONS

SiRNA-based RNA interference (RNAi) is expected to offer an attractive means to sequence-specifically and efficiently silence disease-associated genes for treating various diseases. However, safe and efficient siRNA delivery system is a crucial issue to improve the pharmacokinetic properties of siRNA and promote cellular uptake of siRNA therapeutics. In this study, we have established an arginine-decorated amphiphilic dendrimer **1b** composed of a hydrophobic alkyl chain and a hydrophilic PAMAM dendron bearing arginine terminals as nonviral nanovector for siRNA delivery. Indeed, this dendrimer has conserved the cell penetration advantage of arginine-rich

structural motifs and is able to mediate improved siRNA delivery and potent gene silencing through enhanced cellular uptake of siRNA via arginine structural entities at the dendrimer surface. Our study suggests that decoration of the dendrimer surface with arginine residues is indeed an effective strategy to improve the delivery ability of dendrimers, and the so-constructed dendrimer **1b** is a highly promising nanovector for efficacious siRNA delivery, holding great potential for further therapeutic applications.

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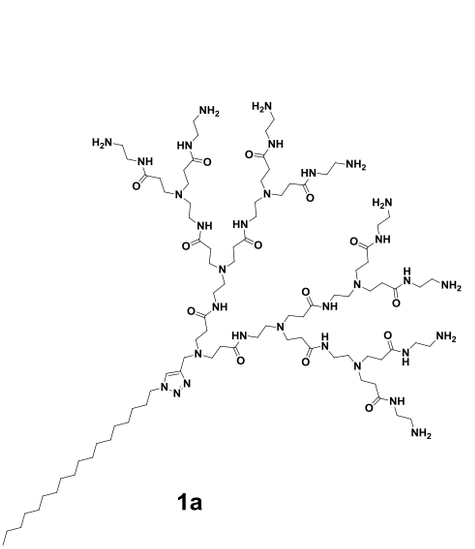
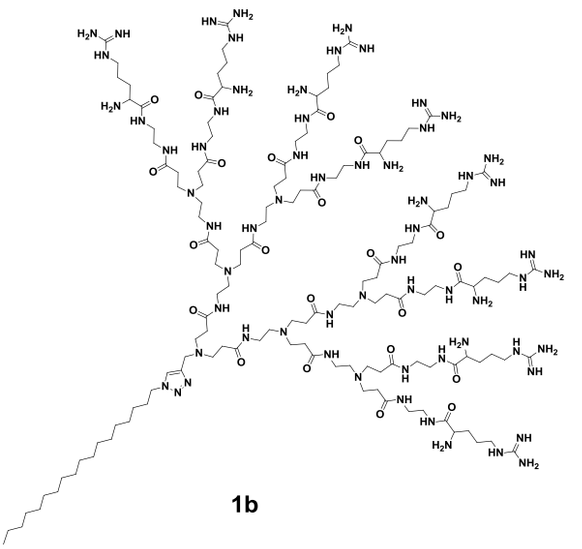
Figure 1. Amphiphilic dendrimers **1a** and **1b** studied in this work.



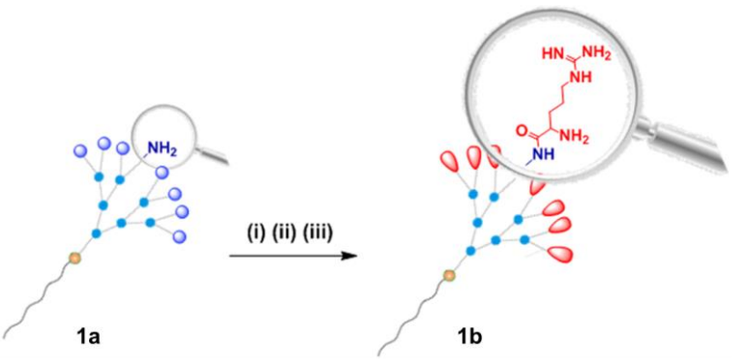
1a



1b

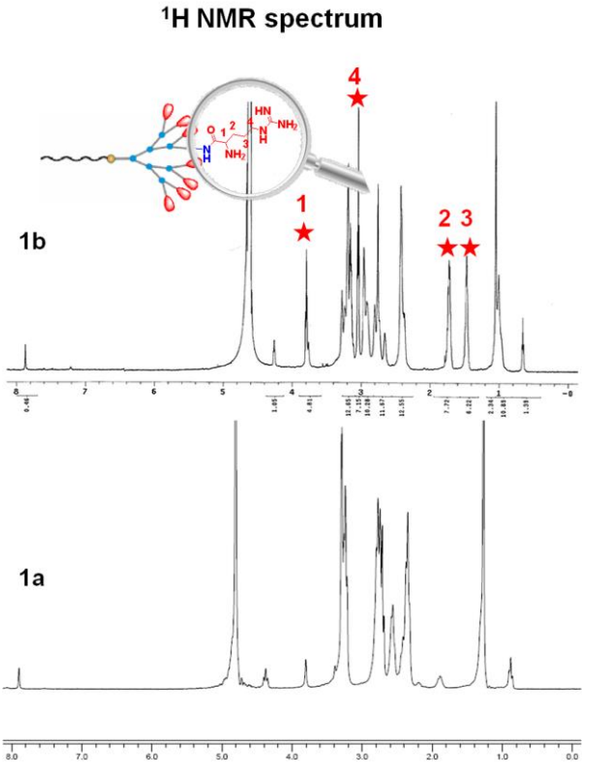
Figure 2. (**A**) Synthesis of the arginine-terminated dendrimer **1b** starting with the amine-terminated dendrimer **1a**. (**B**) ^{1}H-NMR and (**C**) ^{13}C-NMR spectra of **1b** and **1a**. The peaks marked with ★ belong to the characteristic NMR signals of arginine residues.

A



(i) Fmoc-Arg(pbf)-OH, HOBt, HBTU, DIPEA, DMF, r.t.
(ii) 30% piperidine/DMF, (v/v), r.t.
(iii) TFA: triisopropylsilane: H_2O = 95: 2.5: 2.5, (v/v/v), r.t.

B



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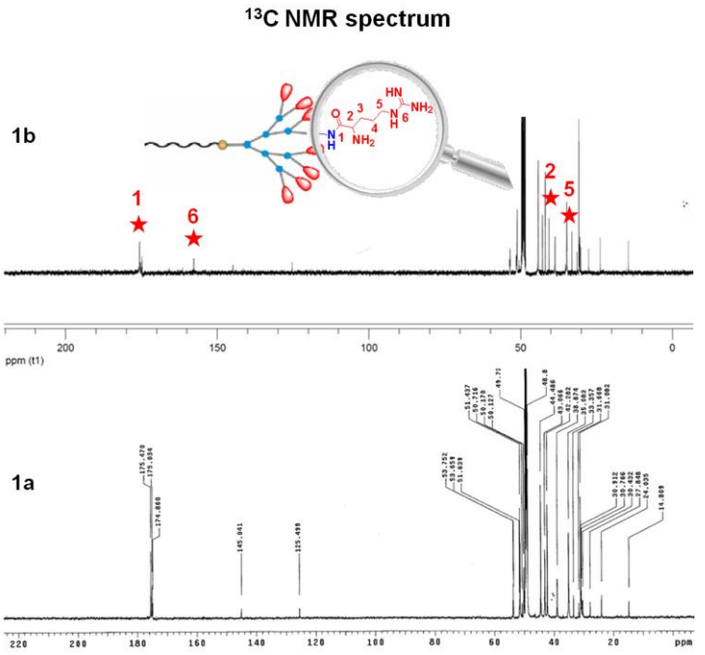
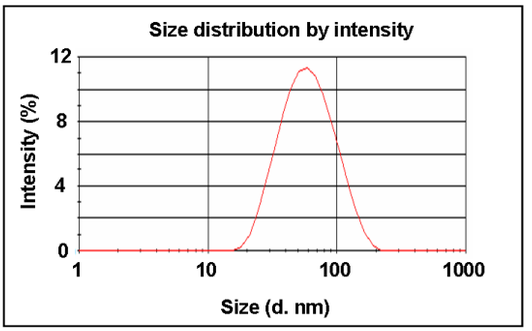
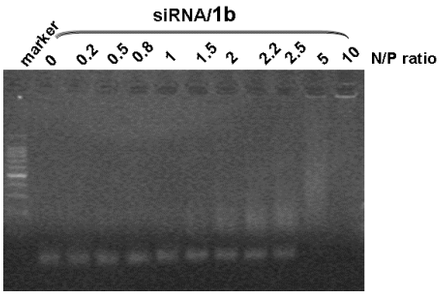


Figure 3. (**A**) DLS analysis of Hsp27 siRNA/**1b** complexes at N/P ratio of 10. (**B**) Migration of Hsp27 siRNA (200 ng/well) in agarose gel in the presence of dendrimer **1b** at N/P ratios varying from 0 to 10. (**C**) Hsp27 siRNA/**1b** complexes (N/P ratio 10) are able to prevent Hsp27 siRNA from RNase digestion.

A



B



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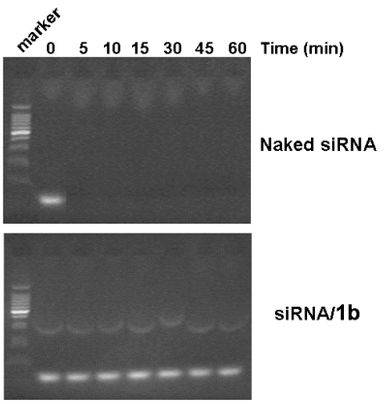


Figure 4. (**A**) Dendrimers-mediated siRNA delivery and gene silencing of Hsp27 in human prostate cancer PC-3 cells at N/P ratio of 10 with 50 nM Hsp27 siRNA, using commercial vector oligofectamine (oligo) as a positive control. *, siRNA/**1a** differs from siRNA/**1b** ($p \leq 0.05$) by Student's *t* test. (**B**) Cytotoxicity profile of **1b** mediated delivery system was determined by MTT assay with **1b** alone and scramble siRNA/**1b** (50 nM siRNA) in PC-3 cells with varying N/P ratios. Experiments were carried out in triplicate. (**C**) **1b**-mediated siRNA delivery and gene silencing of CD4 expression in human hematopoietic CD34+ stem cells at N/P ratio of 5 with 50 nM CD4 dsiRNA. *, siRNA/**1b** differs from control ($p \leq 0.05$) by Student's *t* test. (**D**) Cytotoxicity profile of **1b**-mediated delivery system was determined by MTS assay with **1b** alone, CD4 dsiRNA/**1b** and irrelative dsiRNA/**1b** (50 nM dsiRNA, at N/P ratio 5) in human hematopoietic CD34+ stem cells. Experiments were carried out in triplicate.

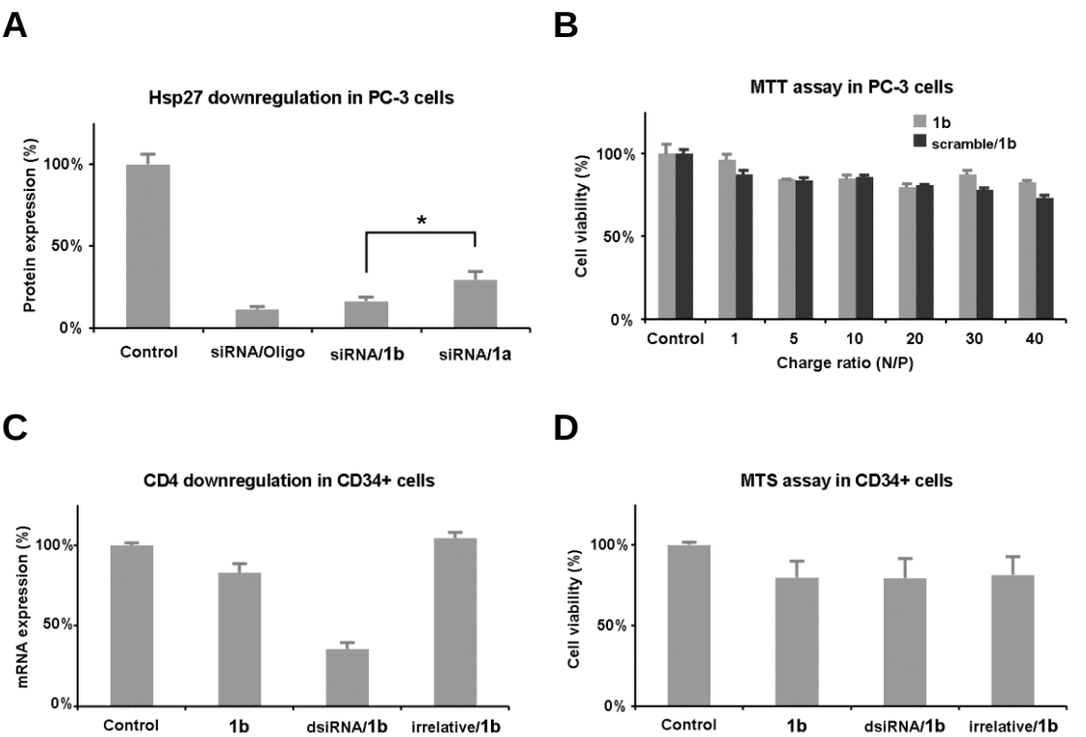
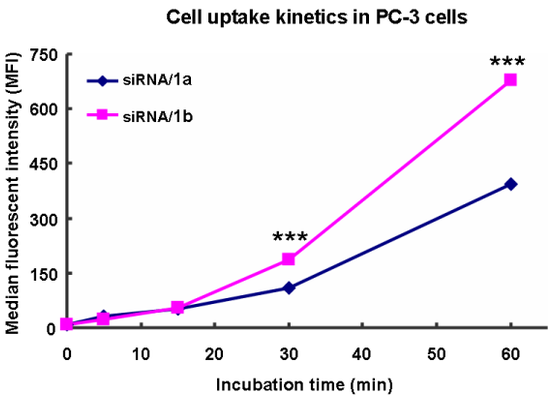


Figure 5. Cellular uptake of the siRNA/**1b** complexes. (**A**) Cell uptake kinetics of the Dy647-labeled siRNA/dendrimers (**1a** or **1b**) complexes (50 nM siRNA at N/P ratio of 10) at different time points in prostate cancer PC-3 cells evaluated using flow cytometric analyses. Experiments were carried out in triplicate. ***, siRNA/**1b** differ from siRNA/**1a** ($p \leq 0.001$) by Student's *t* test. (**B**) Uptake of Cy3-labeled dsiRNA alone, Cy3-labeled dsiRNA/**1b** complexes and Cy3-labeled dsiRNA/**1a** complexes (50 nM siRNA, N/P ratio 5) in human hematopoietic $CD34^+$ stem cells at 24 (left) and 48 h (right) analyzed using a Nikon Eclipse TE2000-S fluorescent microscopy system at 20X magnification. Non-treated cells were used as control.

A



B