

Adaptive Amphiphilic Dendrimer-Based Nanoassemblies as Robust and Versatile siRNA Delivery Systems**

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Abstract: siRNA delivery remains a major challenge in RNAi-based therapy. Here, we report for the first time that an amphiphilic dendrimer is able to self-assemble into adaptive supramolecular assemblies upon interaction with siRNA, and effectively delivers siRNAs to various cell lines, including human primary and stem cells, thereby outperforming the currently available nonviral vectors. In addition, this amphiphilic dendrimer is able to harness the advantageous features of both polymer and lipid vectors and hence promotes effective siRNA delivery. Our study demonstrates for the first time that dendrimer-based adaptive supramolecular assemblies represent novel and versatile means for functional siRNA delivery, heralding a new age of dendrimer-based self-assembled drug delivery in biomedical applications.

Small interfering RNA (siRNA) can efficiently silence disease-related genes in a sequence-specific manner, creating an entirely new siRNA-based gene therapy.^[1,2] However, safe and effective siRNA delivery remains a major challenge.^[2,3] Although viral vectors are very effective, increasing concerns over their safety and immunogenicity substantiate the need to develop alternative nonviral vectors. During the past years, myriads of natural and synthetic nonviral vectors differing in

size, shape, structure, chemistry, and mode of action have been developed for siRNA delivery.^[4–8] These delivery systems can be generally classified into two major groups: lipid and polymer vectors. For both, the efficiency is often dependent on the cell type, with siRNA delivery to primary and stem cells being particularly difficult. Here, we report for the first time an adaptive amphiphilic dendrimer-based nanoassembly acting as versatile vector for effective siRNA delivery and gene silencing, and demonstrate its efficacy for primary and stem cells as well as in vivo. This amphiphilic dendrimer **AD** (Scheme 1 A) self-assembles into vesicle-like dendrimersome nanostructures,^[9] which, in the presence of siRNA, spontaneously undergo structural rearrangement into smaller, spherical micelles, thereby maximizing the interactions between the siRNA and **AD** (Scheme 1 B). The peculiar self-assembly of **AD** into adaptive supramolecular structures alongside its advantageous combination of lipid and dendrimer vector features is evocative of virus-like delivery. Our study hence opens new perspectives in nonviral vector design for siRNA-based gene therapy.

The amphiphilic dendrimer **AD** (Scheme 1 A) was readily synthesized by click chemistry (Scheme S1–S3).^[10] It spontaneously self-assembled in water into spherical nanostructures

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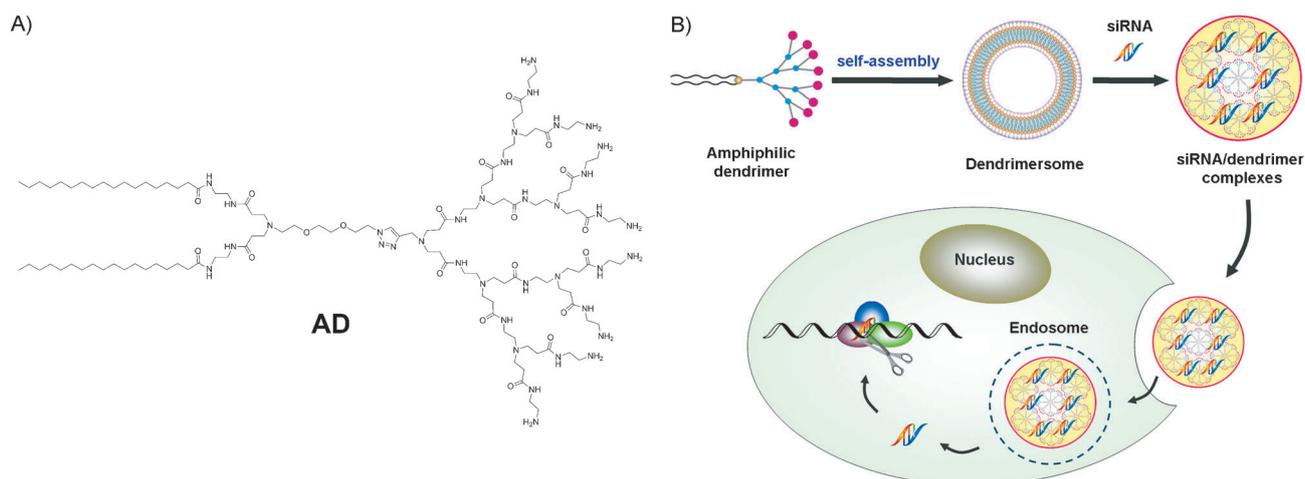
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[**] Financial support from the international ERA-Net EURONA-NOMED European Research project DENANORNA, the Ministry of Science and Technology of China (No.2012AA022501), Agence National de la Recherche (projects DENANORNA and SANAM), Association Française contre les Myopathies (X.L.), Association pour la Recherche sur les Tumeurs de la Prostate (X.L.), La bourse Eiffel (T.Y., M.W.), China Scholarship Council (Y.W., C.C., C.L.), INCa, Canceropôle PACA, Wuhan University, CNRS, INSERM, and the European COST Action TD0802 “Dendrimers in Biomedical Applications” is acknowledged.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201406764>.



Scheme 1. A) The dendrimer **AD** and B) representation of its adaptive self-assembly upon interaction with siRNA for siRNA delivery.

of ca. 200 nm in diameter, as shown by dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Figure 1 A and B). The TEM images showed a corona structure (Figure 1 C), which we inferred to be a bilayer, from the fact that its thickness was 7 nm—approximately twice the theoretical length of **AD** (≈ 3.5 nm). Accordingly, **AD** readily self-assembled to form unilamellar vesicles aptly termed dendrimersomes.^[9] In addition, **AD** formed a Langmuir monolayer film at the air/water interface and could be swelled to form stable giant vesicles (Figure S1), providing further indication of its lipid-like behavior and vesicle-forming properties.

Most interestingly, upon interaction with siRNA, the **AD**-dendrimersomes underwent structural rearrangement to form nano-sized siRNA/**AD** complexes (Figure 1 D) composed of many highly ordered smaller spherical substructures of 6–8 nm diameter (Figure 1 E and F). From this size, which is twice the size of **AD**, we conclude that these substructures are micelles. This vesicular to micellar structural transition is expected to expose more of the positively charged dendrimer surface area, thereby providing stronger stabilizing electrostatic interactions with the negatively charged siRNA. This implies that

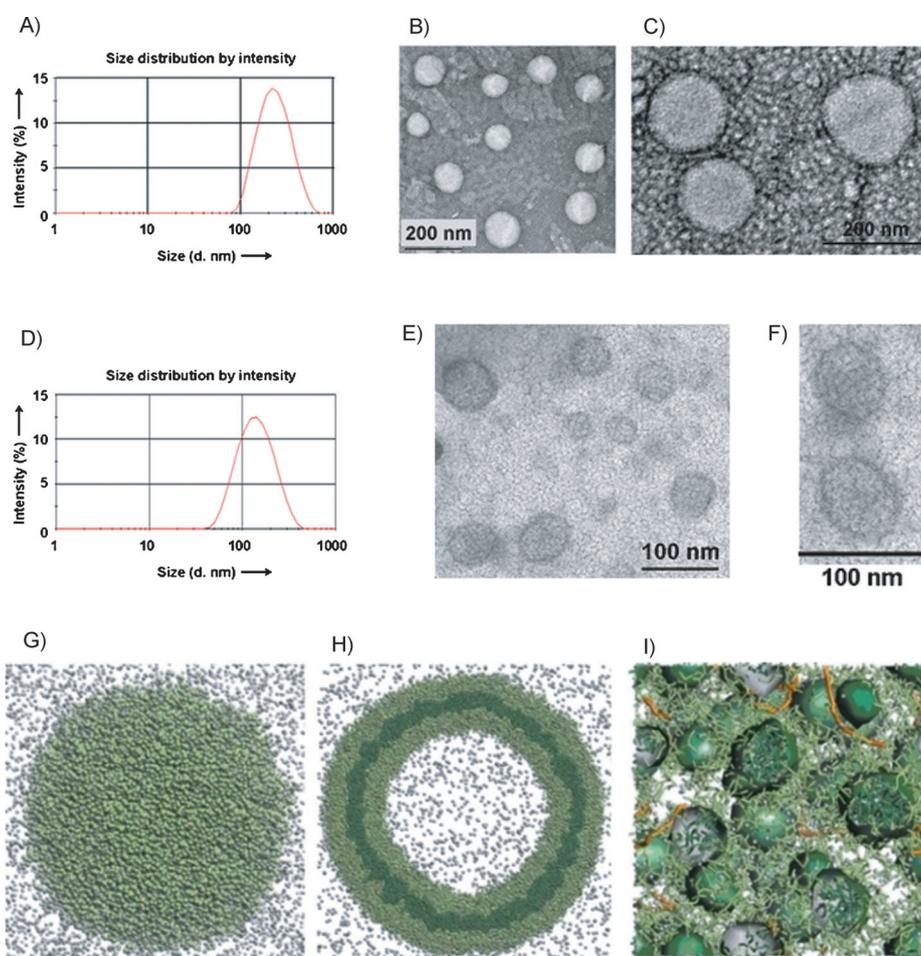


Figure 1. Dendrimer **AD** self-assembles into vesicle-like dendrimersomes, which undergo structural rearrangement into smaller, spherical micelles to interact with siRNA. A) DLS analysis and B,C) TEM imaging of dendrimersomes formed by **AD** in water. D) DLS analysis and E,F) TEM imaging of the siRNA/**AD** complexes. Computer modeling results showing nanostructures formed by **AD** alone (H), a section plane (G), and in the presence of siRNA (I). The hydrophilic units are portrayed in light green, the hydrophobic units in dark green, and siRNAs as orange sticks (see the Supporting Information for more details). Light grey spheres are used to portray some representative water molecules.

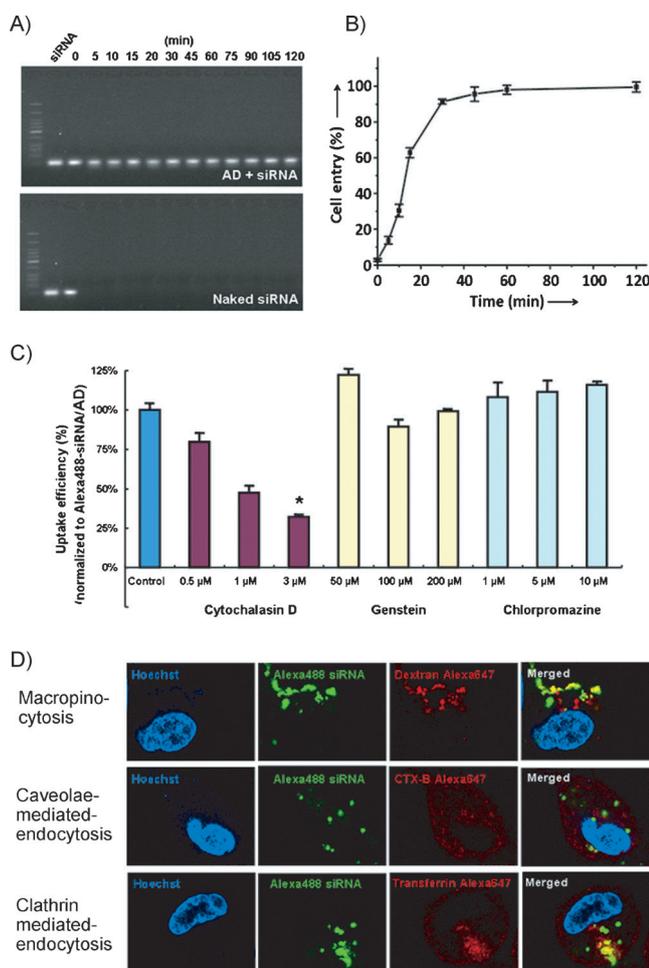


Figure 2. The siRNA/AD nanoparticles are able to A) protect siRNA from RNase digestion and B) promote cell uptake by macropinocytosis (C, D). C) Uptake of Alexa488-labeled siRNA/AD complexes in PC-3 cells in the presence of specific inhibitors and D) confocal imaging of fluorescent markers of different endocytosis pathways. The inhibitors and biomarkers are: cytochalasin D and dextran for macropinocytosis, genestein and CTX-B for caveolae-mediated endocytosis, chlorpromazine and transferrin for clathrin-mediated endocytosis. * differ from control ($p \leq 0.05$) by Student's *t*-test.

AD is able to dynamically self-assemble into responsive and adaptive supramolecular assemblies (Scheme 1B) in the presence of external stimuli.

To verify this hypothesis and gain insight into the adaptive supramolecular assemblies formed by AD, we performed mesoscale computational studies^[11] using dissipative particle dynamics (DPD).^[12] Our simulations show that AD assembles into large supramolecular structures (Figure 1G), whose planar section indeed shows a double-layered dendrimersome (Figure 1H). The formation of the dendrimersome is controlled by the balance of two opposing forces: hydrophobic interactions among alkyl chains that favor cooperative construction of the supramolecular assemblies, and repulsive interactions/steric effects among the positively charged head groups within the dendrons that constrain the assemblies to remain finite sized. Such balance is altered in the presence of siRNA. The conversion of the larger vesicles into smaller

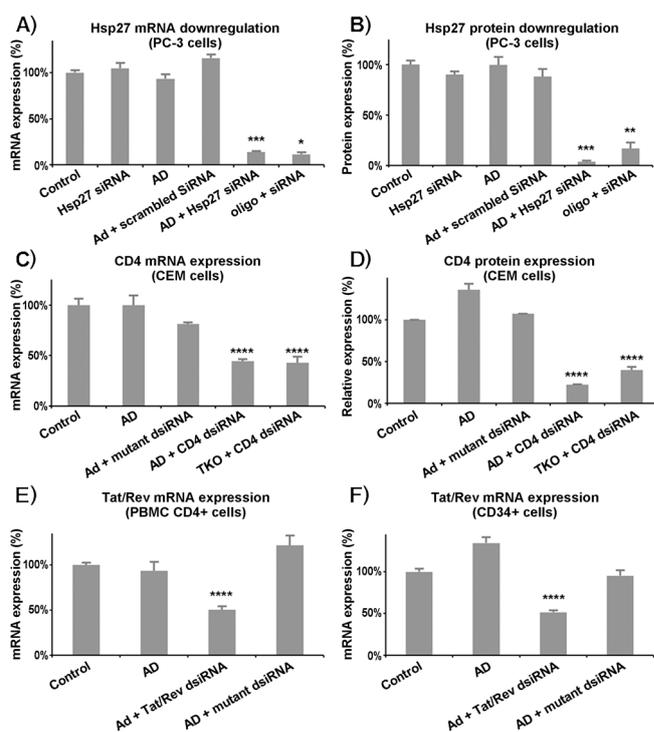


Figure 3. AD-mediated siRNA delivery in human prostate cancer PC-3 cells, human CCRF-CEM T-cells, human primary cells (PBMC-CD4+), and hematopoietic stem cells (CD34+). Gene silencing of Hsp27 at A) mRNA and B) protein levels in PC-3 cells treated with 20 nm siRNA and AD at N/P ratio 10. Down-regulation of CD4 at C) mRNA and D) protein expression on CEM cells treated with 50 nm dsRNA and AD at N/P ratio 5. Knockdown of *Tat/Rev* mRNA expression on E) PBMC-CD4+ cells and F) CD34+ stem cells with 50 nm dsRNA and AD at N/P ratio of 5. Oligofectamine (oligo) and Trans IT-TKO (TKO) were used as controls. *, **, ***, and **** differ from control ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$, respectively) by Student's *t*-test.

micelles (Figure 1I) indeed provides more exposed positively charged surfaces, thus allowing for more efficient electrostatic interactions with the negatively charged siRNA. This in turn results in the formation of stable and compact siRNA/AD nanoparticles in silico.

Indeed, the siRNA/AD complexes are stable colloidal nanoparticles (Figure S2) with a ζ -potential of +25 mV. These nanoparticles could effectively protect siRNA from degradation (Figure 2A) and promote rapid and efficient cell uptake, with almost 100% internalization being attained within 30 min (Figure 2B). Neither the clathrin-mediated endocytosis inhibitor chlorpromazine nor the caveolae-mediated endocytosis inhibitor genestein was able to influence the cellular uptake of the siRNA/AD complexes; conversely, the macropinocytosis inhibitor cytochalasin D significantly decreased their internalization (Figure 2C). Confocal imaging revealed significant co-localization of the siRNA/AD complexes with the macropinocytosis biomarker dextran, whereas little co-localization was observed for either the clathrin-mediated endocytosis biomarker transferrin or the caveolae-mediated endocytosis biomarker CTX-B (Figure 2D). Furthermore, after treatment with siRNA/AD

complexes, actin depolymerization, the hallmark of macropinocytosis, was also observed (Figure S4). Collectively, these findings indicate that macropinocytosis, an advantageous mechanism for drug delivery, is the dominant uptake pathway.

Encouraged by the rapid and favorable cellular uptake results of the siRNA/AD complexes, we went on to assess AD-mediated siRNA delivery in adherent human cancer cells such as prostate cancer PC-3 cells (Figure 3C and D) and breast cancer MCF-7 and MDA-MB231 cells (Figure S5A and B), taking the delivery of Hsp27 (heat shock protein 27)^[13] siRNA as an example. Indeed, a significant attenuation of Hsp27 expression at both mRNA and protein levels (Figure 3A and B) as well as the related anticancer activity (Figure S8) were achieved. Such RNAi efficacy was comparable to or even better than that obtained with the gold reference of oligofectamine (oligo; Figure 3A and B). Remarkably, the silencing effect could be maintained over one week (Figure S7A), even in serum-containing medium in the presence of chloroquine (Figure S7B).

We next demonstrated successful AD-mediated siRNA delivery to suspended human T (CCRF-CEM) and B (Jeko-1) cells (Figure 3C and D, Figure S5C), with dicer-substrate siRNAs (hereafter denoted as dsRNAs)^[14,15] targeting CD4, the primary receptor for HIV-1,^[16] as an example. A considerable decrease of CD4 expression at both mRNA and protein level was achieved (Figure 3C and D). No inhibition was observed with either AD alone or a mutant dsRNA/AD complex. Again, AD was more effective than the relevant commercial reference Trans IT-TKO (TKO; Figure 3D).

The next important challenge was the delivery to primary cells and stem cells. AD was indeed able to exercise functional delivery of RNAi therapeutics to human primary peripheral blood mononuclear cells (PBMC-CD4+) (Figure 3E), hematopoietic stem cells (HSC-CD34+) (Figure 3F), and glioblastoma stem cells (GSCs) (Figure S5D). Following treatment with the anti-*tat/rev* dsRNA delivered by AD, substantial reductions in *tat/rev* mRNA were observed in both the PBMC-CD4+ cells and the HSC-CD34+ cells (Figure 3E and F), leading to more than 50% reduction of viral infection (Figure S9). To our knowledge, this is the first report on the successful and safe delivery of siRNA into primary and stem cells. In fact, the commercially available vector Lipofectamine RNAiMAX (lipo) was

unable to effectively deliver siRNA to human primary and stem cells (Figure S10), whereas Trans IT-TKO (TKO) exhibited notable toxicity on these two cell types.^[17] Our results thus pave the way for the use of AD as a promising vector for future stem cell research and cellular therapy.

To understand the unique and outstanding delivery capability of AD, it was important to explore the delivery mechanism. We hypothesized that, in addition to being able to form supramolecular nanostructures (Scheme 1 and Figure 1), AD might also benefit from the combined advantages of lipid and dendrimer vectors, since AD is in fact a lipid/dendrimer hybrid bearing a hydrophobic alkyl chain entity and a hydrophilic PAMAM dendron.^[10,18] Indeed, the effective delivery ability of AD can be attributed to its peculiar amphiphilic architecture, since neither the hydrophobic alkyl chain **1** nor the hydrophilic dendron **2** alone led to any notable gene silencing (Figure 4A). Importantly, whereas AD could form stable complexes with siRNA and completely retard the migration of siRNA in gel at an N/P ratio of 2.5, dendron **2** alone was not able to do so even at N/P = 10 (Figure 4B). This indicates that the long alkyl chains favor the self-assembly of AD into supramolecular structures, which, in turn, increase the stability of the siRNA/AD nanocomplexes. Interestingly, gene silencing could be significantly enhanced in the presence of the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) which is often employed as a helper lipid to promote the delivery ability of lipid vectors (Figure 4C), underlining the lipid-vector-like character of AD.

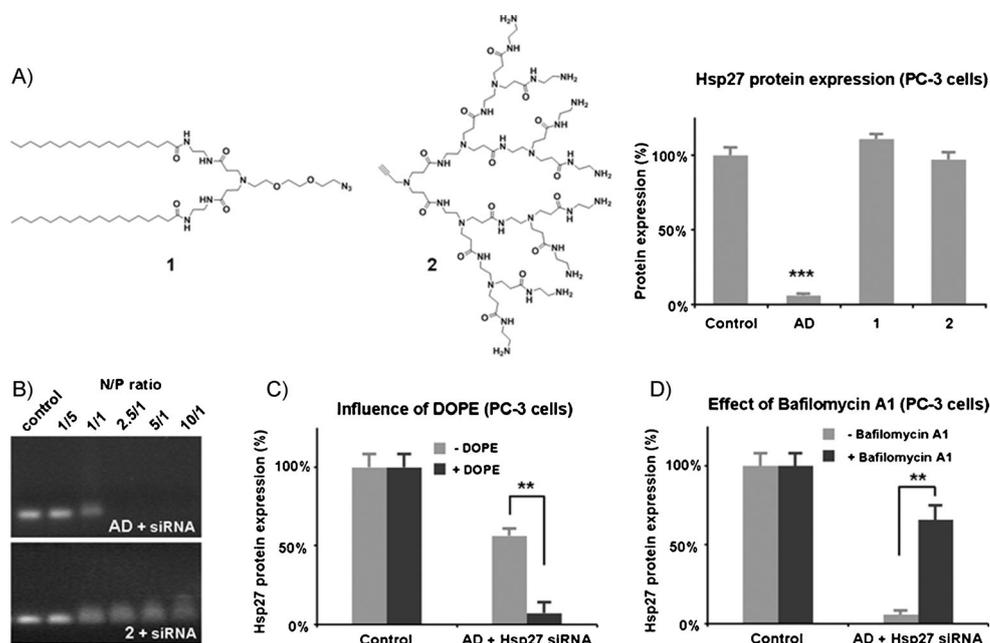


Figure 4. AD-mediated siRNA delivery benefits the advantages of both lipid and dendrimer vectors.

A) Compared to AD, neither the alkyl chain entity **1** nor the dendron **2** led to any gene silencing with 20 nM siRNA at N/P ratio 10. B) Gel retardation of siRNA in agarose gel with AD compared to **2** at varying N/P ratios. C) Dioleoylphosphatidylethanolamine (DOPE) enhanced the gene silencing of Hsp27 with 10 nM siRNA at N/P ratio 10. D) Bafilomycin A1 decreased the AD-mediated gene silencing of Hsp27 observed with 20 nM siRNA at N/P ratio 10. PC-3 cells were used. ** differ from in absence of DOPE or Bafilomycin A1 ($p \leq 0.01$) and *** differ from control ($p \leq 0.001$) by Student's *t*-test.

We further verified whether the so-called “proton sponge effect”,^[19] often invoked to explain the nucleic acid release from the dendrimer complexes, also contributed to **AD**-mediated siRNA delivery. It is believed that inside the acidic endosomal environment, the tertiary amines in the dendrimer interior are protonated, and thus promote endosome disruption and subsequent cargo release in the cytoplasm. Our finding that the gene silencing effect was significantly reduced in the presence of bafilomycin A1 (Figure 4D), a proton pump inhibitor that prevents the acidification of endosomes, implies that **AD**-mediated siRNA delivery was indeed dependent on the endosomal acidification process and that the proton sponge effect played a role in the delivery process.^[20]

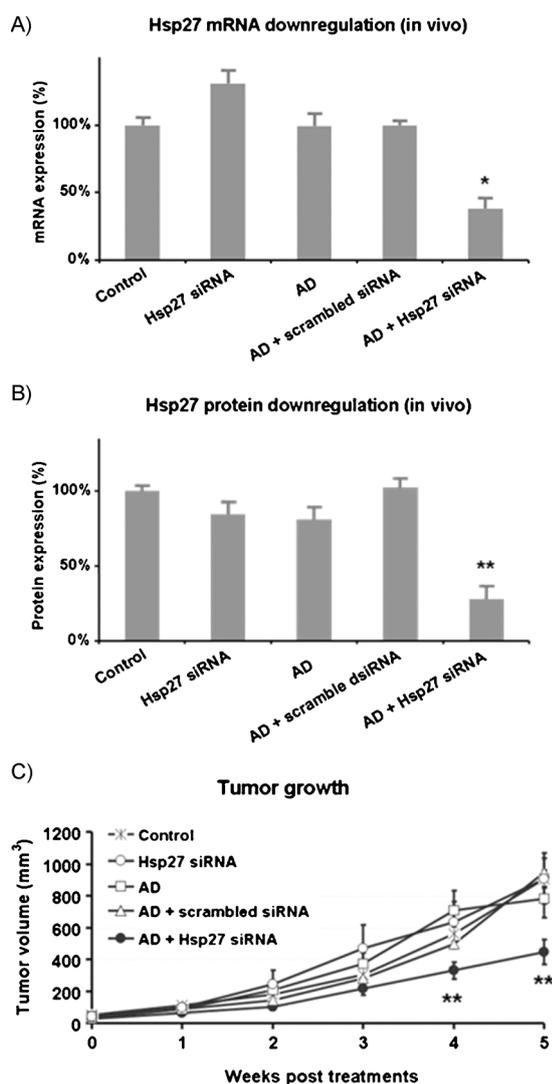


Figure 5. Evaluation of in vivo siRNA delivery using prostate cancer PC-3 xenograft mice following treatment with Hsp27 siRNA/**AD** and the controls of PBS, **AD** alone, siRNA alone, and scrambled siRNA/**AD**, respectively (3 mg kg⁻¹ siRNA and **AD** at a N/P ratio of 5, injection twice a week for 5 weeks). Effective gene silencing of Hsp27 at A) mRNA and B) protein levels was measured using qRT-PCR and western blot, respectively. C) Inhibition on tumor growth assessed by measuring tumor size. * and ** differ from control ($p \leq 0.05$ and $p \leq 0.01$, respectively) by Student's *t*-test.

As a final, decisive step to assess the effective therapeutic potential of **AD** in siRNA delivery, we conducted in vivo delivery of Hsp27 siRNA using a prostate cancer PC-3 xenograft mouse model. Remarkable down-regulation of Hsp27 at both mRNA and protein levels was achieved following treatment with Hsp27 siRNA/**AD** (Figure 5A and B), leading to an effective inhibition of tumor growth (Figure 5C), which is in line with the functional role of Hsp27.^[21] Importantly, there was no discernible toxicity observed since neither weight alteration (Figure S11) nor organ injury nor histopathological changes (Figure S12) were noted.

In summary, here we have presented the novel amphiphilic dendrimer **AD** and its self-assembled adaptive nanostructures as versatile nanocarrier for functional siRNA delivery. **AD**, composed of a positively charged dendron and two lipid-like alkyl chains, combines the advantages of dendrimer and lipid vectors. It readily self-assembles into dendrimer-some nanovesicles which, upon interaction with siRNA, rearrange spontaneously into small spherical micelles. These micelles, by exposing more efficiently the positively charged dendrimer surface, effectively interact, entrap, and condense the negatively charged siRNAs into nanoparticles, which protect the siRNA from degradation. These nanoparticles successfully deliver siRNA into a wide range of cell types including the highly challenging human primary cells and stem cells. They are also efficacious for in vivo delivery. This robust, versatile and nontoxic delivery activity of **AD**, coupled with its easy formulation, yields a promise for a new, highly effective, and cell-type-independent nanovector for efficient and safe siRNA delivery.

Received: July 8, 2014

Revised: August 3, 2014

Published online: September 12, 2014

Keywords: dendrimers · gene silencing · nanocarriers · nonviral vectors · self-assembly

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