**Functional Delivery of siRNA in Mice Using Dendriworms**

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<td>As Published</td>
<td><a href="http://dx.doi.org/10.1021/nn900201e">http://dx.doi.org/10.1021/nn900201e</a></td>
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<tr>
<td>Publisher</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
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<tr>
<td>Accessed</td>
<td>Wed Feb 06 14:45:32 EST 2019</td>
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<td>Citable Link</td>
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Small interfering RNAs (siRNAs) are 21–23 nucleotide double stranded nucleic acid molecules that assemble into specific complexes in mammalian cells to mediate cleavage of complementary mRNA sequences and thus regulate gene expression.1 As a result, their use as a tool for target validation, genetic studies, and for therapy of diseases that are rooted in aberrant gene expression, such as cancer, has become increasingly popular.4 However, unlike small molecule drugs that are often hydrophobic and easily cross the cell membrane, siRNAs are negatively charged and cannot freely traffic to the cytoplasm of the target cells. Thus, delivery of intact, functional siRNAs into the cytoplasm of target cells in vivo is a major impediment to realizing the potential of siRNA-based protein modulation in disease.

To date, several formulations that deliver siRNAs into cells in vitro and in vivo have been reported. siRNA backbone or end-groups modifications that impart siRNA serum stability and improve cell membrane penetration have been used successfully to knockdown genes in vivo.10,11 Protamine-antibody fusions have also been used to successfully transfect HIV infected T cells.12 Lipid-based and lipid-derived nanoparticles that carry siRNAs either in their core or via covalent attachment have been reported to have significant efficacy in vivo and in vitro.13–15 However, these formulations do not contain imaging agents that can be traced in vivo to assess the location of the carrier and siRNAs. To address this issue, many research groups have used quantum dots,16 magnetic17 and gold nanoparticles18,19 as well as carbon nano-tubes20 and enabled successful siRNA/anti-sense delivery in vitro,16,19,20 with a few reports on in vivo imaging of these nanoparticles.17 However, in these reports (where inorganic nanoparticles are used), covalent coupling of siRNAs is used to formulate the nanoparticles which makes rapid testing of various siRNA formulations a cumbersome and iterative process. Thus, to screen for maximum efficacy, it is desirable to have a simple formulation that allows use of different siRNAs in a variety of dosages. Finally, most nanoparticle-based siRNA delivery reports use a model gene to demonstrate knockdown in non-native tissues of animals, which may not be equivalent to a native disease model. Overall, while the above reports highlight significant advances in siRNA delivery, there is a need for a versatile siRNA delivery vehicle that can be traced in vivo, cellular entry, endosomal escape, and knockdown of target proteins.

ABSTRACT Small interfering RNAs (siRNAs) mediate cleavage of specific, complementary mRNA sequences and thus regulate gene expression. Not surprisingly, their use for treatment of diseases that are rooted in aberrant gene expression, such as cancer, has become a paradigm that has gained wide interest. Here, we report the development of dendrimer-conjugated magnetofluorescent nanoworms that we call “dendriworms” as a modular platform for siRNA delivery in vivo. This platform maximizes endosomal escape to robustly produce protein target knockdown in vivo, and is tolerated well in mouse brain. We demonstrate that siRNA-carrying dendriworms can be readily internalized by cells and enable endosomal escape across a wide range of loading doses, whereas dendrimers or nanoworms alone are inefficient. Further, we show that dendriworms carrying siRNA against the epidermal growth factor receptor (EGFR) reduce protein levels of EGFR in human glioblastoma cells by 70—80%, 2.5-fold more efficiently than commercial cationic lipids. Dendriworms were well-tolerated after 7-days of convection-enhanced delivery to the mouse brain and in an EGFR-driven transgenic model of glioblastoma, anti-EGFR dendriworms led to specific and significant suppression of EGFR expression. Collectively, these data establish dendriworms as a multimodal platform that enables fluorescent tracking of siRNA delivery in vivo, cellular entry, endosomal escape, and knockdown of target proteins.

KEYWORDS: dendrimer, siRNA, nanoparticle, EGFR, glioblastoma

*Address correspondence to sbhatia@mit.edu.
Received for review March 1, 2009 and accepted August 4, 2009.
Published online August 12, 2009. 10.1021/nn900201e CCC: $40.75
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VOL. 3 • NO. 9 • 2495–2504 • 2009
AcsNano
that is nontoxic, allows flexible siRNA loading, and can be used in vivo in a native disease model.

In this work we report synthesis, systematic in vitro evaluation, and in vivo application of polyvalent, dendrimer-bearing, magnetic nanoparticles (dendriworm) as a carrier for siRNA delivery in a transgenic murine model of glioblastoma. The construct overcomes issues highlighted above. First, dendriworms contain magnetic core and are labeled fluorescently to enable imaging in a variety of formats. Second, we establish that dendriworms promote cytosolic release of the endocytosed cargo more efficiently than their components, resulting in efficient delivery of siRNA to the cell cytoplasm over a wide range of loading doses. Finally, noncovalent attachment of siRNA affords dendriworms the ability to retain flexibility in siRNA loading without reformulation and enabled a smooth transition to in vivo applications where dendriworms were well tolerated after 7 days of convection-assisted delivery in the CNS and resulted in knockdown of EGFRvIII in a transgenic model of glioblastoma. In the future, this multimodal platform may be further enhanced to include affinity ligands for targeting to particular cells of interest.

RESULTS AND DISCUSSION
Polyamidoamine (PAMAM) dendrimers are cationic polymers that have been extensively evaluated as candidates for gene delivery. Dendrimers are branched, multivalent macromolecules that were first reported nearly two decades ago. Dendrimers can be synthesized to have neutral, cationic, or anionic groups in a cascade polymer form, enabling facile regulation of a number of functional groups, size, and charge. Multiple surface groups on dendrimers can be linked with polymers, amino acids, tissue targeting moieties, drugs, nucleic acid molecules, or imaging agents to modulate properties of the dendrimer-based drug or imaging agent carriers.

Synthesis and Characterization of Dendriworms. While dendrimers have been used for delivery of genes and antisense oligonucleotides, delivery of siRNA with dendrimers has been challenging and only poor efficiencies have been reported. A recent report demonstrates use of generation 7 PAMAM dendrimers for siRNA delivery, but most efforts using lower generation dendrimers for siRNA delivery have been unsuccessful and it has been shown that lower generation dendrimers are poor at forming uniform, stable particles with siRNAs. In a recent report, generation 6 polylysine dendrimers were also found to be inefficient for delivery of siRNAs. Since dendrimers buffer the endosomes via their tertiary and secondary amines, we hypothesized that polyvalent conjugation of lower generation dendrimers onto an elongated magnetic nanoparticle host could generate a construct that would induce high proton sponge effect and enable efficient endosomal escape of siRNAs. Use of lower generation dendrimers may also diminish the likelihood of immune system activation and inflammatory response associated with the use of higher generation dendrimers. Also, to encourage further in vivo use, we chose a magnetic nanoparticle scaffold that could be conjugated to fluorophores or imaged via magnetic resonance imaging.

The synthesis of dendrimer-conjugated magnetic nanoworms (dendriworms) is shown in Figure 1A. First,
generation 4, cystamine core PAMAM dendrimers were reduced in 0.5 M dithiothreitol (DTT), and excess DTT was removed using a G25 chromatography column. Next, amine-modified, cross-linked iron oxide nanoworms, labeled with near-infrared fluorochromes (VivoTag 680, Visen Medical, Inc.), were prepared as detailed in the Experimental Section. Finally, reduced dendrons and nanoworms were conjugated using the heterobifunctional linker N-succinimidyl 3-(2-pyridyldithio)-propionate, which links amine groups on the nanoparticle with the central thiol on reduced dendrons (Figure 1A; see Experimental Section for details). The resulting dendriworms were separated from free dendrons by filtering several times using either 100,000 molecular weight cutoff filter (Amicon Ultra, Millipore Corp.), magnetic separation column (MACS, Miltenyi Biotec), or G50 chromatography columns. Dendriworms were stored at 4 °C protected from light and were stable for at least 6 months. Net charge on dendriworms was found to be between 16–24 mV (zeta-potential measurement in 1× PBS) and the average hydrodynamic diameter was found to be between 80 and 110 nm (dynamic light scattering measurements in 1× PBS, Zetasizer Nano, Malvern, Inc.). In the transmission electron micrograph, the particles were found to retain a worm-shape, where multiple 5 nm iron oxide cores were lined in a series (data not shown).43 The number of dendrons per dendriworm was found to be 45–50 using fluorescencem and ninyhydrin tests (assuming there are approximately 7 iron cores per dendriworm). Further, using UV–vis absorbance measurements, the number of VivoTag on each dendriworm was found to be between 1 and 5. A key feature of the dendriworms is that dendrons are conjugated via a reducible linker (SPDP) to the nanoparticle surface. This implies roughly 50 siRNA molecules loaded on each dendriworm (there are roughly 7 iron cores per dendriworm). To maintain consistency, amounts of dendriworm and nanoworm are expressed in iron core concentration in the rest of the manuscript. The stability of the siRNA-dendriworm nanoparticle was assessed in fetal bovine serum (FBS) and artificial cerebrospinal fluid (ACSF) by gel electrophoresis and dynamic light scattering. To visualize the residual bound siRNA, 1% SDS was used to release the siRNA prior to electrophoresis. In ACSF the nanoparticle and the bound siRNA remained stable for up to 6 h (Supporting Information, Figure S1); however, the siRNA showed lower stability in serum (data not shown).

**Dendriworms Enable Efficient Release of Cargo into the Cell Cytosol.** Cationic polymers adhere to the negatively charged moieties on the cell membrane and may be taken up via fluid phase endocytosis or via receptor-mediated endocytosis.44 As a result, a cargo that is only active in the cell cytoplasm and is delivered using cationic polymers or cationic nanoparticles needs to escape the endosomes. To test our hypothesis that conjugating multiple cationic dendrimers on a nanoworm scaffold would enhance the proton sponge effect, leading to better cytosolic release of cargos, we evaluated the efficiency of dendriworm endosomal escape compared to free dendrimers. To assess endosomal escape, we codelivered excess of a membrane impermeable dye (Calcein at 0.25 mM) along with the siRNA delivery agent (dendrimer, unmodified nanoworms and dendriworms) coupled with siRNAs and counted the fraction of cells with endosomal escape as indicated by diffuse cytoplasmic distribution of the dye.45,46 The results of these endosomal escape experiments are shown in Figure 2. Aminated nanoworms (200 nM iron core), dendrimer (30 μM), or dendriworm (200 nM iron core) were incubated with 100 nM siRNA in serum-free cell culture medium containing excess of Calcein for 10 min at room temperature and transferred over the cells. The cells were kept at 37 °C and 5% CO2 environment for 1 h and imaged using an inverted microscope. In the presence of Calcein alone, punctate cytoplasmic distribution of dye was consistent with the endosomal uptake. Some degree of endosomal release as judged by diffuse cytoplasmic distribution was seen with aminated nanoworms. Significantly more endosomal escape was seen with both dendrimers and dendriworms, although the extent of endosomal escape was much greater with dendriworms. Endosomal escape is
also dependent on uptake of the dendrimers or dendriworms into the endosomes which depends on primary amines on the surface of dendrimers that are positively charged in serum (near pH 7). Thus, addition of more negatively charged siRNAs that neutralize the positively charged primary amines may reduce the number of dendrimers or dendriworms that are able to load into the endosomes, affecting the number of buffering (tertiary) amines per endosome available for inducing endosomal escape. Indeed, when higher concentrations of siRNA were mixed with dendrimers or dendriworms, dendrimers failed to induce significant endosomal escape while dendriworms remained effective even when incubated with 1 μM siRNA (Figure 2B,C). Thus conjugation of several dendrons (reduced half of a dendrimer) onto aminated nanoworms allows for more effective endosomal escape.

Next, we examined the cytotoxicity of dendriworms at concentrations used in our studies. Two types of toxicity assays relying on either mitochondrial reductase enzyme activity (MTT assay, based on reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or on intracellular esterase activity (Calcein AM assay) were used. No significant in vitro toxicity across various dendriworm and siRNA concentrations used in this report was observed (Supporting Information, Figure S2).

**Dendriworms Deliver siRNA into Cells and Induce Gene Silencing.** We have shown that dendriworms are able to deliver cargo into cells efficiently at concentrations that have negligible toxic effects on cells in vitro. Next, we examined whether dendriworms could be used to deliver siRNAs into human primary glioblastoma cells (GBM-6) that express mutant EGFRvIII, and induce gene silencing. EGFRvIII gene silencing (knockdown) in glioblastoma cells was assessed by measuring EGFR protein expression levels and the amount of mRNA transcripts compared with levels of a housekeeping gene (GAPDH) (Figure 3). Both protein and mRNA levels were suppressed significantly and were comparable to the commercial transfection agent Lipofectamine 2000 when 100 nM siRNA was delivered. Greater relative (dendriworm vs Lipofectamine 2000) gene silencing was observed at protein level than at mRNA level. One potential cause for this could be the dependence of mRNA read-out method (qRT-PCR) on several rounds of amplification, which may attenuate the differences if the initial number of mRNAs is different but high in two samples. Gene silencing was found to be strongly dependent on siRNA and dendriworm dose (Figure 3). The knockdown was also sequence dependent as lamin siRNA did not lower the expression levels of EGFR (Figure 3). Further, EGFR siRNA delivered using aminated nanoworms or dendrimers did not produce significant EGFR knockdown (Supporting Information, Figures S4, S5). In addition to GBM-6 cells, dendriworms also produce significant gene knockdown in HeLa cells (Supporting Information, Figure S3) although, consistent with the literature on the variability of polymeric formulation dependent siRNA delivery, the extent of knockdown in HeLa cells is lower. Taken together these results imply that dendriworms are a highly effective siRNA delivery vehicle for GBM-6 glioblastoma cells and represent a flexible for-
mulation due to noncovalent siRNA attachment for optimization of siRNA delivery to other cell types.

**Systemic Delivery of Dendriworms.** We evaluated the utility of dendriworm-based siRNA delivery in mice. When dendriworms (20 μg of iron in 100 μL of 1 × PBS) were injected in the bloodstream via tail vein injections, preferential accumulation in the lungs, as expected from the cationic nature of the particles, was seen (Supporting Information, Figure S6). Briefly, Swiss Webster mice were injected in the tail vein with fluorescently labeled dendriworms and the amount of fluorescence remaining in the blood at various time points was measured. This was done by drawing blood at different time points, removing cells and debris via centrifugation at 800 rpm for 5 min, and measuring the fluorescence from the supernatant using an Odyssey infrared imaging system (Li-Cor Biosciences, Inc.). In this experiment, dendriworms cleared the blood within 5 min (Supporting Information, Figure S9).

To further probe where the particles were accumulating, 100 μL of 1 × PBS containing 20 μg of dendriworms or 20 μg of fluorescently labeled CLIO-NH2 or 1 × PBS alone was injected in the tail vein of mice and the mice were sacrificed after approximately 10 min of injections. After further 10 min, the organs were removed, washed with saline, and imaged with Odyssey infrared imaging system (Li-Cor Biosciences, Inc.). Since dendriworms were labeled with infrared emitting dye VivoTag680, fluorescence could be used to infer the degree of uptake of particles in various organs. Images were color coded to reflect the intensity of uptake (Supporting Information, Figure S6, left). After observing significant accumulation in the lungs (also confirmed using histology; data not shown), we sought to test if the accumulation was lung-specific. To test this, one pair of Swiss Webster mice was injected with PBS and the other pair of mice was injected with dendriworms, in the left heart ventricle. The results from this experiment are shown in the right side of Figure S6. The results show that dendriworms still accumulate in the lungs and the reticuloendothelial filtration organs.

Therefore, we sought to use dendriworms in vivo via means that do not depend on systemic circulation of the particles to reach the target organ or tissue (e.g., intraperitoneal cavity or intravenous injections).

Figure 3. Delivery of EGFR siRNA into glioblastoma cells with dendriworms. (A) EGFR protein levels were assessed using Western blot with GAPDH as a housekeeping gene. Lamin siRNA was used as a control for testing sequence specificity and Lipfectamine 2000 was used as a positive control. EGFR/GAPDH expression ratio was plotted from at least three independent experiments. (B) EGFR to GAPDH mRNA transcript level ratio was calculated using qRT-PCR. Concentration of dendriworm expressed in iron core concentration. The asterisk (*) represents a p-value of <0.05.
Convection-Enhanced Intracranial Delivery of EGFR siRNA Using Dendriworms Reduces EGFR Expression in GBM Tumors.

Given the efficacy of RNA interference in GBM cells in vitro, we studied the effects of siRNA delivery by dendriworms to the brain parenchyma. First, we determined the biodistribution within CNS tissues of healthy CD-1 outbred mice, by formulating 0.5 mg/mL fluorescently labeled dendriworms with 0.115 mg/mL siRNAs intracranial infusion for a period of 3 days and 7 days using an osmotic pump delivering particles at a rate of 0.5 μL/h.

Particles were observed in tissue sections from both the posterior and the anterior locations with respect to the injection site for both the 3 days delivery (Figure 4B, C, and D) and 7 days delivery (Supporting Information, Figure S7). We observed that the nanoparticles were able to penetrate throughout the brain parenchyma in a distance-dependent fashion as reflected in greater fluorescence intensity levels in the tissue sections closer to the site of injection and detectable nanoparticles at the far border (Figure 4B). Dendriworms were found to be present at the perinuclear region in cells in vivo (Figure 4C, D and Supporting Information, Figure S7) consistent with the particles entering the cells. Histopathological evaluation of the injected animals revealed no discernible pathology at the concentration used (Supporting Information, Figure S7B), confirming the lack of carrier-induced toxicity from dendriworms. These data suggest applicability of dendriworms for siRNA delivery into CNS tissues via sustained, convection-enhanced delivery. On the basis of these results, a concentration of 0.5 mg/mL of dendriworm was chosen for further in vivo studies.

To test dendriworms’ delivery efficacy in vivo, we used a genetically engineered mouse model of glioblastoma, in which expression of oncogenic EGFR variant III leads to de novo GBM tumor formation. At 20 days post tumor initiation, VivoTag 680 dye-labeled dendriworms carrying an EGFR siRNA or a GFP siRNA or aminated nanoworms carrying EGFR siRNA were delivered intratumorally in brain tumor bearing animals by convection-enhanced delivery using an osmotic pump for a period of 7 days at 0.5 mg/mL of dendriworm and 0.115 mg/mL of siRNA (11 μg total siRNA). Post infusion, animals were sacrificed and immunofluorescence was performed on the tumor sections to analyze EGFR knock down. The images and the results from quantification of EGFR expression are shown in Figure 5 (also see Supporting Information, Figure S8). In the animal treated with dendriworms carrying EGFR siRNA, strong and significant suppression of EGFR expression was seen (Figure 5A, D) compared with the animal treated with dendriworms carrying GFP siRNA (Figure 5B, D) or the aminated nanoworms mixed with EGFR siRNA (Figure 5C, D). On the other hand, similar amount of nanoparticles were taken up in the brain across the three treatments (Figure 5D). In fact, even with slightly higher nanoparticle uptake in the control samples, the level of EGFR expression was much higher than in the...
animal treated with the dendriworm + EGFR siRNA formulation. Furthermore, distinct mismatch in location of nanoparticles and EGFR expression was seen in the sections from dendriworm + EGFR siRNA treated samples while significant overlap and even colocalization of nanoparticles with EGFR was seen in the other control samples. Using a relevant de novo GBM model system whereby tumor cells grow and migrate through the microenvironment similar to what is observed in human patients is necessary for ascertaining the penetrating power of dendriworms. Our data demonstrate that dendriworms are not only able to diffuse into brain tumors but can also deliver siRNA into the tumor cells, resulting in significant gene silencing in vivo. In this context, these results reveal that the use of dendriworms for siRNA delivery to GBM may have a clear clinical impact on this disease.

CONCLUSION

In this work, we demonstrate a systematic design-based approach to achieve siRNA delivery, both in vitro and in vivo, using dendriworms. We also demonstrate the correlation between the extent of endosomal escape and the efficiency of gene silencing. The marked enhancement in endosomal escape efficiency of dendriworms compared to dendrimers or nanoworms suggests that enrichment of polymers along the nanoworm backbone promotes improved proton sponge effect and endosomal escape, resulting in better siRNA silencing. This paradigm can be applied to other gene delivery polymers to design polyvalent, nanoparticle-based siRNA delivery agents that are effective across various cell lines and amenable to multimodal in vivo detection and tracking.

Using dendriworms, we also demonstrate significant suppression of EGFR expression levels in glioblastoma cells and in vivo in a transgenic mouse model of glioblastoma. This finding has clinical significance because glioblastoma multiforme is an especially aggressive form of cancer often with elevated expression levels of EGFR. Further, inhibition of EGFR activity has been demonstrated to be clinically beneficial in a subset of patients implying that elimination of EGFR signaling through gene expression knockdown may result in positive clinical outcomes.

The combination of delivery, imaging, and targeting capabilities within dendriworms offers a versatile and powerful agent to combat malignant glioma. Availability of several primary amine groups on dendriworms makes them an attractive platform for conjugating other entities that can modulate their tumor uptake, biodistribution, and tissue homing properties. The magnetic core in dendriworms should also allow in vivo imaging of dendriworms via MR. Finally, while several methods for the delivery of nanotechnology-based therapeutics for treatment of malignant gliomas have been developed, in most cases, these entities offer a single modality of detection or delivery and have proven to be toxic at functional concentrations, which is in contrast.

Figure 5. In vivo knockdown of EGFR expression in transgenic mice. (A) EGFR siRNA delivered with dendriworms, (B) GFP siRNA delivered with dendriworms, (C) EGFR siRNA delivered with nanoworm-NH₂, and (D) quantitative analysis of EGFR expression per cell or nanoparticle (NP) uptake relative to number of cells (DAPI) using data from 6 separate sections from a mouse for each treatment (all images included in the Supporting Information). See Experimental Section for quantification details. Scale bar is 100 μm.
with the low neuro-toxicity we observed with dendriworms. Thus, in conclusion, dendriworms are po-

tent siRNA delivery nanoparticles for in vivo applications and offer an unprecedented versatility.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** Unless noted otherwise, all chemical were purchased from Sigma-Aldrich, Inc. All nonproprietary siRNAs were obtained from Dharmacon, Inc.

**Synthesis of Dendriworms.** Cross-linked iron oxide nanoparticles were synthesized using published methods.35,42 Briefly, 4.5 g of Dextran (M, 15000 – 25000) and 0.63 g of FeCl3 · 6H2O were dissolved in 10 mL of cold, sterile water in a round-bottom flask purged with nitrogen and kept in an ice bath. Freshly prepared solution of 0.25 g FeCl3 · 4H2O in cold 5 mL sterile water was injected into the flask while the solution was stirred at 800 rpm. Next, 1 mL of cold ammonia was added into the flask dropwise. After 10 min the reaction was allowed to proceed at 80 °C for 1 h. The resulting reaction product was diluted 1:4 in sterile water and filtered (Amicon Ultra-4 MWCO: 100 000). Finally, the par-
ticles were filtered using 0.2 μm, 32 mm syringe filters (Pall Cor-
poration, East Hills, NY) to remove large particles and aggregates. A 1.5 mL aliquot of particles was cross-linked by adding a mixture of 2.5 mL of 5 M NaOH and 1 mL of epichlorohydrin and al-
lowing the reaction to proceed by stirring at room temperature for 24 h. Resulting particles were dialyzed using 50 000 MWCO membrane and aminated overnight by adding 10% ammonia.

Cross-linked, aminated iron oxide particles were activated with 500-fold excess of SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate, Pierce Biotechnology, Inc.) and reacted with VivoTag ag680 (Visen Medical, Inc.). The particles were filtered using a G50 (GE Healthcare) sephadex column. Next, Cystamine core, PAMAM dendrimers were reduced using 0.5 M DTT (dithiothre-
tol) by reacting at room temperature for 2 h. Excess DTT was re-
moved using G25 column and reduced PAMAM dendrons were

**Particle Characterization.** Concentration of nanoparticles was measured as iron core concentration by measuring absorbance at 400 nm. VivoTag concentration was measured using absorbance at 670 nm, and Dendron concentration was estimated by reacting at room temperature for 2 h. Next, the sample was centrifuged at 4 °C for 1 h, rinsed in PBS, and serial coronal sections were

**Endosome Escape Assay.** Membrane impermeable dye Calcein (0.25 mM) or FITC-conjugated dextran (10 000 MW) were code-
livered with the various nanoparticle formulations. After 1 h, ex-
cess dye was washed off, and cells were visualized with a confocal

diagnosis imaging system. Ratio of number of cells with endosome escape (indicated by a diffuse cytoplasmic fluorescence) and to-

tal number of cells counted from the phase contrast image was used to quantify the results.

**Toxicity Assessment.** MITT and Calcein AM assays were per-
formed using HeLa cells in 96-well plates. Nanoparticle formula-
tions at various concentrations were incubated with the cells for 4 or 24 h. Cells were then washed three times and the reagents were added. For MTT assay, the absorbance was read at 570 nm after 4 h. For Calcein AM assay, cells were incubated with Calcein AM for 30 min and a fluorometer was used with 485 nm ex-
citation and fluorescence was read at 530 nm. Data were ana-
lyzed using Graph Pad Prism 3.03.
sense strand: 5′-GG CUA CGU CCA GGA GCG CAC C-3′) cells were left in the cell culture incubator for 48 h. For EGFR knockdown assessment, cells were incubated for 72 h. After the incubation period, cells were trypsinized and resuspended in 1× PBS containing 1% fetal bovine serum. Reduction in GFP expression was measured using flow cytometry; EGFR protein and mRNA levels were measured using Western blot and quantitative RT-PCR (Bio-Rad iCycler). Primary antibodies for Western blots were purchased from Cell Signaling (EGFR antibody catalog no. 22232 and GAPDH antibody catalog no. 2118). Western blots were developed using HRP-secondary antibody along with the SuperSignal West Pico substrate (Pierce Biotechnology, Inc.). Primers for EGFR and GAPDH mRNA amplification and quantification via qRT-PCR were obtained from Integrated DNA Technologies: (EGFR forward primer) TAGAATTCTATCGCG-GCGAGACCA, (reverse primer) TACGCGTGACCTGCAAGAAG; (GAPDH forward primer) TCGACAGTCAGCCGCATCTGCAGGACCA, (reverse primer) TGACAGTCCGACCCTACTCTTC, (reverse primer) ACCAATACGTGACTGCACTCT.

Acknowledgment. We thank Greg Underhill for help with Q-RT-PCR, Sandra March for help with the western blots, Marga- rita Akiyama with toxicity analysis, Erika Batchelder of the Keck imaging facility at the Whitehead Institute at MIT for help with transmission electron micrographs, and Muthiah Manoharan and colleagues for help with the helpful discussions. A. A. acknowledges the generous support from the Pierre Casimir-Lambert Fund. This work was supported in part by the Casimir-Lambert Fund.

Supporting Information Available: Figures S1 – S9. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

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