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Synthesis and Functional Evaluation of DNA-Assembled Polyamidoamine Dendrimer Clusters for Cancer Cell-Specific Targeting

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Summary

We sought to produce dendrimers conjugated to different biofunctional moieties (fluorescein [FITC] and folic acid [FA]), and then link them together using complementary DNA oligonucleotides to produce clustered molecules that target cancer cells that overexpress the high-affinity folate receptor. Amine-terminated, generation 5 polyamidoamine (G5 PAMAM) dendrimers are first partially acetylated and then conjugated with FITC or FA, followed by the covalent attachment of complementary, 5′-phosphate-modified 34-base-long oligonucleotides. Hybridization of these oligonucleotide conjugates led to the self-assembly of the FITC- and FA-conjugated dendrimers. In vitro studies of the DNA-linked dendrimer clusters indicated specific binding to KB cells expressing the folate receptor. Confocal microscopy also showed the internalization of the dendrimer cluster. These results demonstrate the ability to design and produce supramolecular arrays of dendrimers using oligonucleotide bridges. This will also allow for further development of DNA-linked dendrimer clusters as imaging agents and therapeutics.

Introduction

Ideal antitumor therapeutics might have multiple functions, such as targeting to a tumor, imaging the presence of the tumor, and delivering a therapeutic to tumor cells [1]. Polyamidoamine (PAMAM) dendrimers are promising candidates for use as the backbone of multitasking therapeutics because of their well-defined surface functionality, low polydispersity, good water solubility, and lack of immunogenicity [2–4]. Recently, controlled surface modification followed by conjugation of folate (FA) and fluorescein (FITC) moieties on the surface of the dendrimer has yielded molecules capable of targeting to tumor cells through folate receptors [5]. Conjugating these multiple small molecules to a single dendrimer surface, however, may result in synthetic problems such as decreased water solubility and low yield due to the hydrophobicity of the attached functional groups and the steric hindrance of the densely packed dendrimer surface [5, 6]. Also, using a single dendrimer as targeted drug delivery platform would require different targeted drugs for each varied tumor type or molecular alterations in each specific cancer cell.

Recently, we have hypothesized that the self-assembly of PAMAM dendrimers using complementary single-stranded oligonucleotides might be a versatile manner of constructing common, combinatorial anticancer therapeutics [7]. Synthetic oligonucleotides have been widely used as a tool to self-assemble nanoscale objects in precise structural arrangements due to the base specificity of the resulting duplex structure [8–10]. Oligonucleotides could be conjugated to dendrimers with single functional molecules requiring only two conjugation steps, which would allow for construction of combinatorial libraries of dendrimer-linked anticancer therapeutics, imaging, and targeting agents. In the current study, we test the ability of this unique dendrimer platform to target to cancer cells overexpressing folate receptor by conjugating targeting (FA) and imaging (FITC) molecules to two different dendrimers, which are then linked with complementary oligonucleotides (5′-phosphate-modified 34-base-long oligonucleotides; 34b). The prototype DNA-assembled nanocluster of the FA-conjugated dendrimer and FITC-conjugated dendrimer was evaluated in vitro to test tumor cell-specific binding and internalization.

Results and Discussion

Characterization of the molecular properties of the dendrimer conjugates and their supramolecular assemblies made from these components is indispensable for the effective design and use of DNA-assembled dendrimer complexes [11]. In this regard, monitoring of the synthesis of the assembly using exacting analytical techniques is of great importance. The entire synthesis of the cluster agent consists of three conjugation reactions and one hybridization reaction (Figure 1). The theoretical size and shape of the target structure was predicted by using Insight II software (Accelrys, San Diego, CA) to be 20 nm in diameter, with an 11 nm DNA linker (Figure 2A). Chemical and structural characterization of the initial starting material (PAMAM dendrimer generation 5; G5 PAMAM) and the surface-modified dendrimers were performed using complementary analytical techniques such as ultraviolet-visible (UV-vis), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), size exclusion chromatography-multiangle laser light scattering (SEC-MALLS), reverse phase high-pressure liquid chromatography (RP-HPLC), and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to determine the purity, average molecular weight, polydispersity, and surface functionality of the dendrimers and dendrimer conjugates.

Characterization of Partially Acetylated G5 PAMAM Dendrimer

The acetylation reaction of the amine-terminated dendrimer is reported to be very efficient and stoichiometri-

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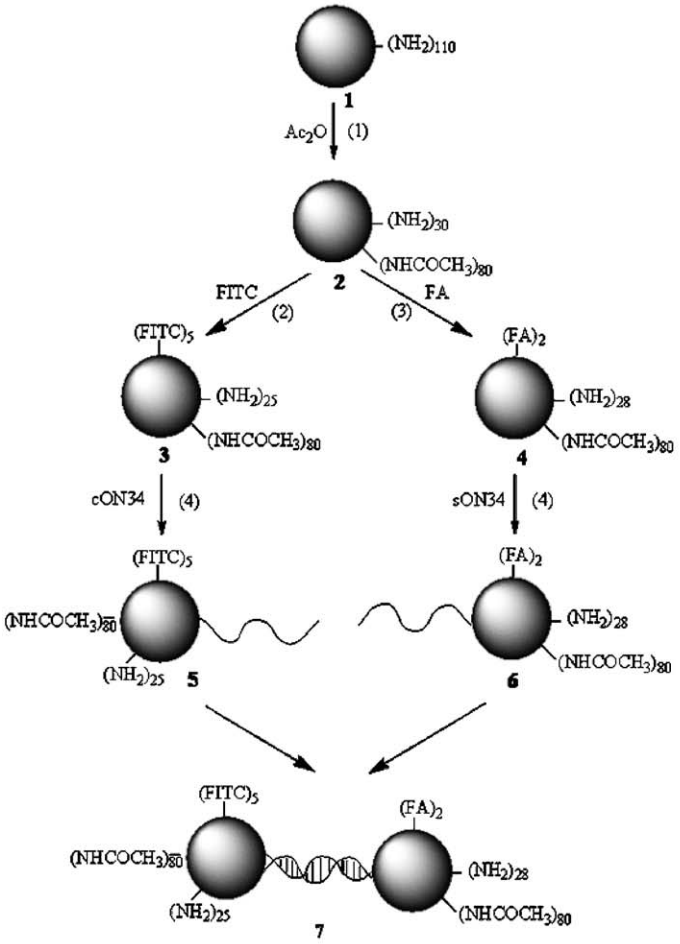

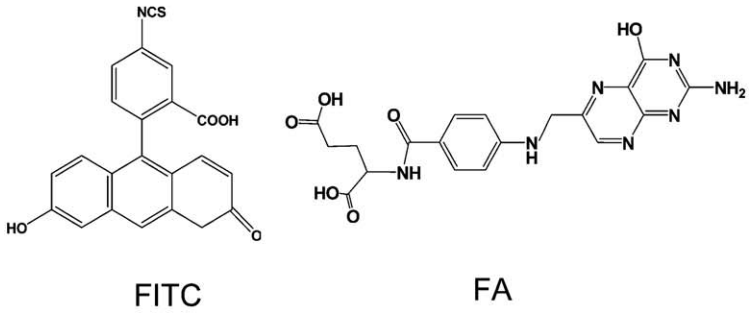

Figure 1. Synthetic Scheme for Preparation of DNA-Linked Cluster of G5-FITC and G5-FA Dendrimers

(1) Triethylamine, MeOH, 16 hr; (2) DMSO; (3) EDC in DMF:DMSO (3:1, v/v); (4) 0.1 M EDC/0.1 M imidazole (pH 6.0) in 0.5 M LiCl; (5) 10 mM phosphate buffer (pH 7.4), 150 mM NaCl, annealed at 90°C 10 min then cooled to room temperature over 3 hr.

cally controllable [12]. To determine the substoichiometric equivalent of acetic anhydride to the dendrimer, the number of the terminal primary amine groups on the dendrimers was first determined to be 110 (as compared to the theoretical value of 128) by potentiometric titration and SEC-MALLS measurement. This result is consistent with other findings explaining the inherent structural defects from incomplete reactions or other problems in the divergent synthesis of the dendrimer, causing missing repeating units, intramolecular cyclization, dimer formation, and retro-Michael reaction in dendrimers [13–15]. The addition of 82 molar equivalents of acetic anhydride to the terminal primary amine groups of the dendrimer in the presence of triethylamine resulted in partial conversion of the primary amine groups to acetamide moieties. The removal of the acetic acid, which is a byproduct of the acetylation, was facilitated by extensive dialysis of the reaction mixture against a phosphate-buffered saline (PBS) solution (pH 7.4). The degree of acetylation was measured by ^{1}H

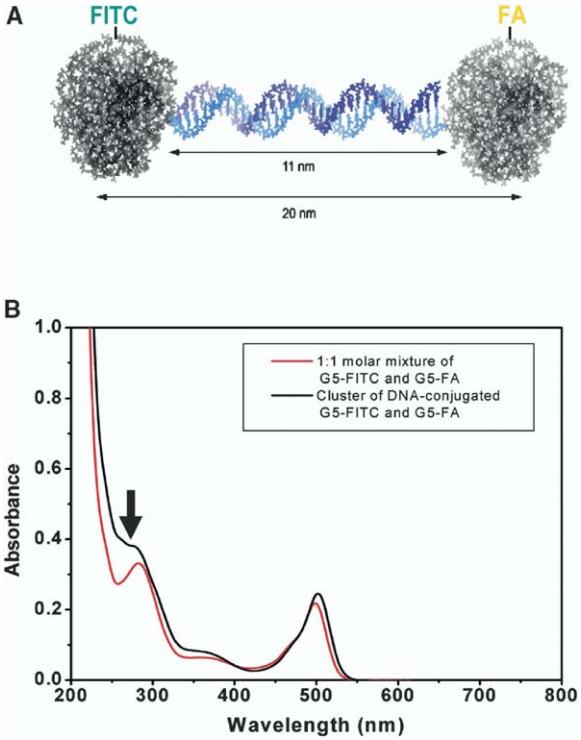

Figure 2. Molecular Modeling and UV-Vis Spectroscopy

(A) Prediction of overall dimension of the desired DNA-linked functional dendrimer cluster by using Insight II software. The DNA linker (34 bp) is 11 nm and the center-to-center distance of the two functional dendrimers is estimated to be 20 nm. FITC and FA are schematically shown on the periphery of the dendrimer. (B) UV-vis spectra of the DNA-linked G5-FITC and G5-FA dendrimer cluster recovered after 100 kDa MWCO membrane filtration. The arrow indicates the presence of DNA in the cluster, which appears as a shoulder peak at 260 nm. For comparison purpose, the equimolar mixture of the G5-FITC (3 μM) and G5-FA (3 μM) dendrimers in PBS is shown as a red line.

NMR, using the specific acetyl proton peak at 1.9 ppm. The increasing intensity of this signal indicated that an average of 80 acetyl groups were added to each dendrimer (G5-Ac_{80}-$[NH_2]_{30}$). The yield of this reaction was 85% by weight (782 mg), and the calculated molecular weight of the final product was 28,752 g/mol. This result was consistent with the M_n (28,600 g/mol) obtained from SEC (Table 1). The product showed a polydispersity similar to the intact, amine-terminated dendrimer starting material (1.038 versus 1.01, respectively). Further evidence of the successful partial acetylation came from MALDI-TOF, which shows a broad peak at 29,562 ($[M + H]^+$) and 15,400 ($[M + H]^{2+}$).

Characterization of FITC-Conjugated G5 PAMAM Dendrimer

FITC was qualitatively documented to be conjugated to the dendrimer by thin layer chromatography (TLC) analysis, which showed that FITC conjugated to the dendrimer was being retained at the origin while unconjugated free FITC migrated at the solvent front (R_f = 0.72). The approximate number of conjugated FITC molecules per G5 dendrimer was determined by UV and ^{1}H NMR spectroscopy. The UV absorption peak of the conjugate appeared at 502 nm, shifting 10 nm from the peak of free FITC (λ_{max} = 492 nm), which is an indication of conjugation of the dye to the dendrimer. In contrast, simple mixing of the acetylated dendrimer and FITC did not cause a shift in the absorption peak (see Supplemental Figure S6). The absorbance at 502 nm shows a linear relationship with the concentration of the conjugate; extinction coefficient = 246,600 M^{-1} cm^{-1}. From a calibration curve of the free dye, the average number of FITC molecules per dendrimer was calculated to be 5 ± 0.4. This result was consistent with ^{1}H NMR spectroscopy evaluation using the sum of the integrals of aromatic protons of FITC.

Characterization of FA-Conjugated G5 PAMAM Dendrimer

The condensation reaction between the active ester of folic acid and the remaining primary amines on the partially acetylated dendrimer was conducted in the presence of water-soluble carbodiimide (1-ethy-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; EDC). The UV spectrum of the conjugate dissolved in water at a concentration of 2 nM showed a maximum peak at 280 nm and a broad shoulder at 370 nm. From a calibration curve of free FA, the number of FA molecules was calculated to be 2 ± 0.4 when five molar equivalents of FA to the dendrimer were used in the conjugation reaction. This low level of folate on the dendrimer may be attributed to the undesired reaction of the two carboxylic acid groups (α-, γ-) of the folic acid and the two amine groups of the dendrimer, even though the γ-carboxylic group is more labile to EDC activation [6]. From the integral ratio of aromatic protons in FA molecules (8.6, 8.0, 7.6, 6.7 ppm) to the signal at 1.9 ppm which corresponds to the methyl protons in the acetamide groups

Table 1. Molecular Properties of PAMAM Dendrimers Used for Oligonucleotide Conjugation

Sample Code	Simplified Sample Formula	M_w (g/mol)	PDI[b]
G5[a]	G5-$(NH2)_{110}$	26,962	1.068
G5-Ac	G5-Ac_{80}-$(NH2)_{30}$	29,562	1.031
G5-Ac-FITC	G5-Ac_{80}-$(NH2)_{25}$-$(FITC)_5$	30,700	1.045
G5-Ac-FA	G5-Ac_{80}-$(NH2)_{28}$-$(FA)_2$	32,505	1.017

[a] The number of the terminal primary amine groups was determined by potentiometric titration, where the dendrimer (10 mg) dissolved in 0.1 N NaCl solution was first fully protonated by the addition of a 0.1 N HCl standard solution, followed by titrating with 0.1 N NaOH standard solution at 3 min intervals at 20°C. Theoretical number is 128.

[b] Polydispersity index (PDI) is defined as number-average molecular weight (M_n) divided by weight-average molecular weight (M_w) from SEC data.

on the surface, the average number of FA moieties in the conjugate was calculated to be 2.5 ± 0.5 molecules of FA residues per dendrimer, which agrees well with the UV result.

Phosphate-Modified DNA Oligonucleotide Conjugation to PAMAM Dendrimers

Synthetic DNA has been used as a linker to assemble nanoscale objects [9, 10, 16, 17]. Furthermore, the attachment of DNA oligonucleotides to organic or inorganic polymers, such as proteins and metallic nanoparticles, has been extensively studied [18–20]. Recently, the covalent attachment of oligonucleotide to either the core or the periphery of the dendron of various dendrimers has been reported, where SH-modified oligonucleotides or SH-core dendrons are coupled to a maleimide group present in a linker or an oligonucleotide to produce the oligonucleotide conjugates [21, 22].

We employed phosphoramidate chemistry developed by Chu et al. [23] between the 5′-phosphate group of the single-stranded DNA and the terminal amine group of the PAMAM dendrimer to covalently attach a synthetic oligonucleotide DNA to the dendrimer surface. The activation of the phosphomonoester (which is more nucleophilic than internucleotidic phosphodiesters) by a water-soluble carbodiimide (EDC) in the presence of a catalyst (imidazole) allows the intermediate to react in situ with primary aliphatic amines of the dendrimer surface (see Supplemental Data for the reaction scheme).

However, given the potential for interaction of the positively charged amine-terminated dendrimer and the negatively charged DNA, this charge interaction must be prevented to avoid insoluble complex formation [23, 24]. Thus, the partial capping of the surface primary amine groups with acetamide groups was also necessary to minimize electrostatic interactions at physiological pH. In addition, the use of molar excesses of either dendrimer or DNA in conjunction with salt (LiCl) prevented charge-based complex formation between the dendrimer and the DNA. As we have reported earlier and suggested by DeMattei et al. [7, 22], both minimizing the nonspecific interaction between DNA and dendrimer and limiting the number of DNA on each dendrimer are crucial to constructing dendrimer components into a nanostructure with defined size and shape.

In this study, we used a five-molar excess of dendrimer to DNA so that the number of DNA conjugated to the dendrimer would be limited to approximately a single strand per dendrimer. In this way, any unreacted dendrimer was removed after the hybridization reaction of the DNA-conjugates by centrifugal filtration using a 100 kDa molecular weight cutoff (MWCO) membrane which retains the desired DNA-linked functional dendrimer nanocluster. Because the cluster is assumed to have a dumbbell-like structure with a theoretical molecular weight of approximately 86 kDa, this method allows for separation of the target nanostructure from the unlinked globular FITC-conjugated or FA-conjugated dendrimers or the unhybridized DNA-dendrimer components.

Figure 2B shows UV-vis spectra of the retentate and the filtrate of the hybridization reaction mixture of DNA

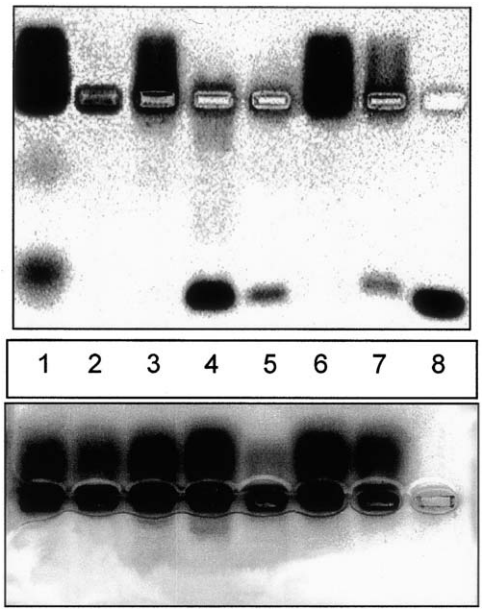

Figure 3. Identification of Dendrimers and DNA Using Agarose Gel Electrophoresis

The functional dendrimers (lanes 1 and 2), oligonucleotide-conjugated functional dendrimers (lanes 3 and 4), DNA-linked dendrimer cluster (lane 5), mixture of the functional dendrimers (lane 6), and mixture of oligonucleotide-conjugated functional dendrimers (lane 7) were run in the 2% agarose gel prepared in Tris-borate-EDTA buffer (1× TBE; pH 8.0) and stained simultaneously with EtBr (upper panel) and colloidal blue (lower panel). Lane 1: G5-Ac-FITC (2.6 mg/ml in 0.5 M LiCl); lane 2: G5-Ac-FA (0.8 mg/ml in 0.5 M LiCl); lane 3: G5-Ac-FITC-c34d (1 mg/ml in PBS); lane 4: G5-Ac-FA-s34d (1 mg/ml in PBS); lane 5: hybridized G5-Ac-FITC-c34d and G5-Ac-FA-s34d in PBS; lane 6: mix of lanes 1 and 2; lane 7: mix of lanes 3 and 4; lane 8: oligonucleotide (c34d, 0.5 μg).

(34b)-conjugated FA-dendrimer and FITC-dendrimer using the diafiltration method (5000 × g, 15 min). Absorbance at 260, 354, and 500 nm indicates the presence of DNA, FA, and FITC, respectively. From the absorbance change at 500 nm, we found that approximately 80% of the G5-FITC dendrimer permeated the membrane, indicating that the rest of the G5-FITC dendrimer was retained as a result of the clustering with the G5-FA dendrimer via a complementary DNA hybridization reaction. The extinction coefficient we obtained for G5-FITC dendrimer allowed us to calculate the concentration of the cluster assuming that the dimeric nanocluster has an approximate molecular weight of 86 kDa.

The dendrimer conjugates and the DNA-linked clusters were also characterized by agarose gel electrophoresis (Figure 3). Because of the remaining 20 primary amines (after partial acetylation), the acetylated dendrimer is slightly positively charged. In the case of G5-FITC dendrimer, even after extensive dialysis and gel filtration, FITC entrapped in an internal void of the dendrimer branch structure is still identified in lane 1. In the conjugation mixture, a large amount of free dendrimer persists. While no free DNA was detected in lane 3 (G5-FITC-c34), free DNA was observed in lane 4 (G5-FA-s34). This indicated a potential difference in reactivity of oligonucleotides with the two functionalized

dendrimers. Simultaneous staining with ethydium bromide (EtBr) and colloidal blue showed colocalization of the dendrimer and DNA, suggesting oligonucleotide-conjugated functional dendrimers which have net negative charges due to the negatively charged oligonucleotides attached to the dendrimers. In lane 5 (a duplex hybrid formed from the G5-FITC-c34 and G5-FA-s34), some free DNA was still present even after YM-100 membrane filtration. However, this free DNA was removed after a second membrane filtration. This indicates that the direct conjugation of DNA to dendrimer via phosphoramidate bonds may not be efficient enough, probably due to limited accessibility of the oligonucleotide to the densely packed surface of the dendrimer which has free amine groups available for the 5′-phosphate group of the DNA after FITC or FA conjugation. This steric effect might be overcome through the application of a limited number of heterobifunctional linker molecules prior to reaction with the DNA.

Biological Evaluation of the DNA-Linked Functional Dendrimer Cluster Agent

FA is known to be internalized into cells through a high-affinity, receptor-mediated process [25]. The high-affinity receptor for FA is overexpressed on a number of human tumors, including cancers of the ovary, kidney, uterus, testis, brain, colon, lung, and myelocytic blood cells [26–29]. The KB cells we employed for this study are a human epidermoid carcinoma cell line that overexpresses folate receptors, especially when grown in low-folic acid medium. Because the affinity of folate conjugates for the cell surface folate receptors is high ($K_D \sim 10^{-10}$ M), FA-modified dendrimers allow the specific delivery of diagnostics and therapeutic agents to cancer cells in the presence of normal cells. Thus, the conjugates of folic acid linked to either a single drug molecule or an assembly of molecules can bind to and enter receptor-expressing tumor cells via folate-mediated endocytosis [5, 30].

The binding of the DNA-linked dendrimer nanocluster to KB cells showed concentration-dependent uptake, with saturation occurring at approximately 40 nM (Figure 4A). The sigmoidal profile of the dose-dependent curve suggests that the uptake of the dendrimer cluster is saturable, indicating the binding of the cluster to the KB cells overexpressing folate receptors with an apparent affinity of at least 15 nM to achieve 50% of maximal binding. The saturation of the binding curve occurred at 40 nM, which is comparable to the binding capacity (200 nM) of the single-dendrimer conjugate [5]. This binding capacity of the cluster allowed for a 200% increase in the mean fluorescence measured at 500 nm after incubation for 1 hr at 37°C with a concentration of 40 nM. In addition, increases in cell fluorescence from the binding of the DNA-linked dendrimer nanocluster were effectively blocked by the addition of free FA in the medium. A free FA concentration of 5 μM reduced the cell fluorescence from the cluster device by more than 90%, indicating that the binding event of the cluster is mediated by the folate receptor (Figure 5). The relative rapid uptake, its characteristic dose dependency, and the antagonism by free FA suggest that the uptake of the cluster of the FITC-dendrimer and FA-dendrimer linked by DNA was mediated by folate receptors of the KB cells.

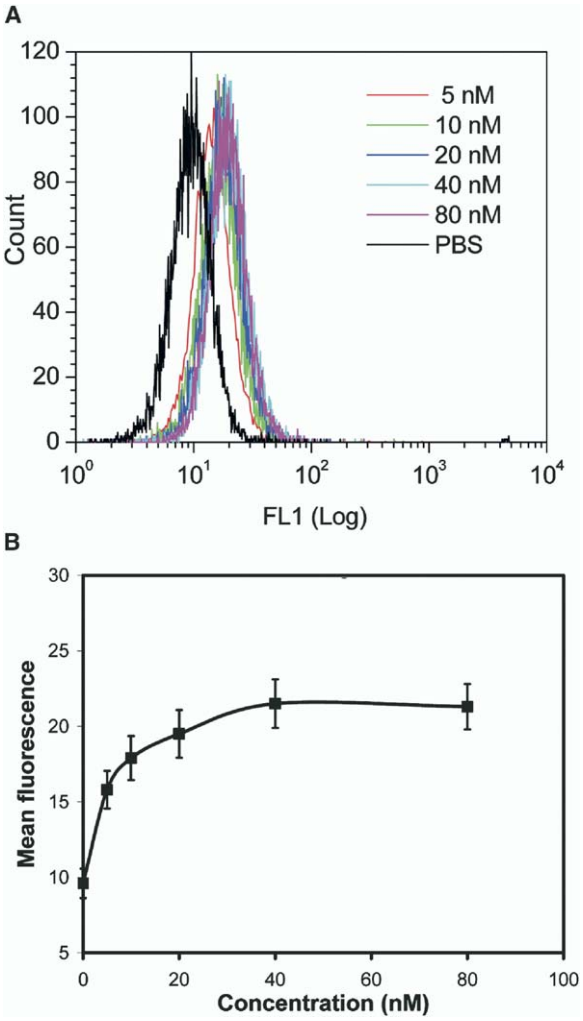

Figure 4. Binding of DNA-Linked Functional Dendrimers to KB Cells after 1 hr Incubation

(A) Gradual increase of cell-associated fluorescence after incubation with increasing concentrations of DNA-linked G5-FITC and G5-FA dendrimer cluster agent. (B) The concentration-dependent saturation curve indicates specific binding of the DNA-linked cluster to KB cells overexpressing folate receptor.

We also used several control samples to show that the cell-associated FITC fluorescence originated from the DNA-linked FITC-dendrimer and FA-dendrimer clusters rather than from a nonspecific aggregate of the two functional dendrimers (without regard to the presence of a duplex DNA linkage). The mixture of the DNA-conjugated dendrimer which did not undergo the annealing process bound poorly to the KB cells. The DNA-conjugated G5-FITC dendrimer (G5-FITC-cON34) alone did not show significant binding, suggesting that the nonspecific binding of the G5-FITC-cON34 is negligible (Figure 5C). These control experiments strongly imply that the nonspecific association of the DNA-conjugated functional dendrimers with the cell surface is not the cause of the cell bindings and uptake we observed.

The binding observed in the flow cytometry studies was further confirmed in confocal microscopic images of the cells (Figure 6). A significant difference of fluores-

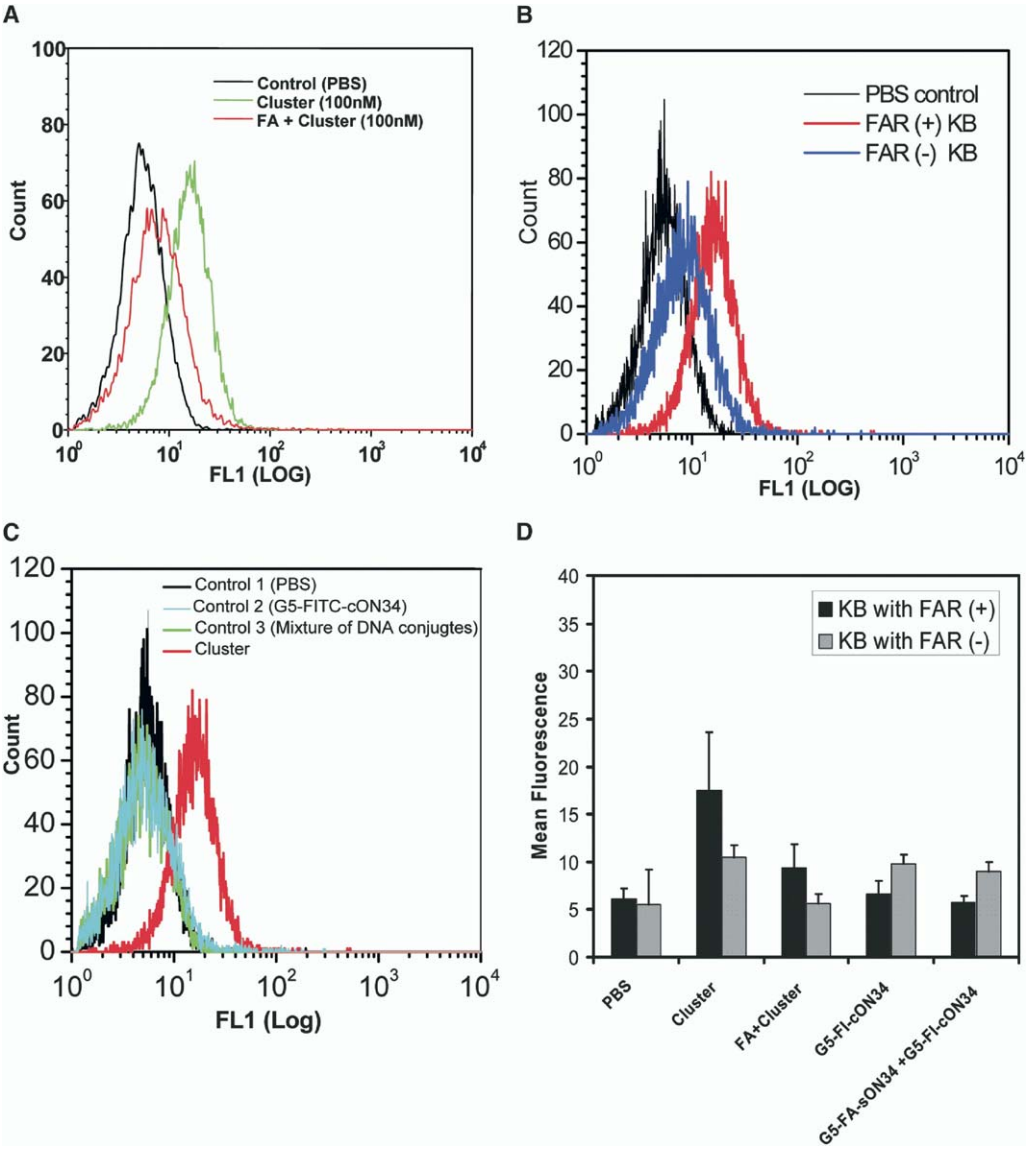

Figure 5. Evidence for Specific Binding of the DNA-Linked Dendrimer Cluster Agent to KB Cells via Folic Acid Receptor (FAR)

(A) Competitive inhibition of the cluster agent (100 nM) by free FA (5 μM). (B) The cluster failed to bind to the folate receptor-downregulated KB cells (FAR–). (C) Control groups (2: G5-FITC-cON34; 3: mixture of G5-FA-sON34 and G5-FITC-cON34 with no annealing process) showed no nonspecific binding, suggesting a clustering of dendrimer-DNA conjugates did not occur in vitro without in situ DNA hybridization reaction. (D) Comparison of the mean fluorescence intensity of the cluster and the cluster antagonized by free FA, and controls (G5-FITC-cON34 and the mixture of the DNA conjugates).

cence from the PBS control was not obtained due to the low concentration of the DNA-linked cluster agent (40 nM) as well as the autofluorescence of the control cells in the measured emission wavelength (515 nm). However, the confocal analysis found the presence of the cell-associated fluorescence of the DNA-linked dendrimer cluster both in the periphery of the cell membrane and at the cytoplasm of the cell, suggesting the clusters had been internalized across the membrane into the cell.

We have demonstrated the targeting function of the DNA-linked dendrimer cluster agent. Synthesis, characterization, and the functional evaluation of this material were performed and confirmed the reaction efficiency and the purity of the cluster, as well as documenting receptor-medicated specific targeting and cellular uptake by the cluster. The biological findings also indirectly confirm that the two different functional dendrimers were self-assembled by DNA heteroduplex formation. Further studies will be focused on constructing multifunctional dendrimer therapeutics targeted to different cancer cells, using DNA-directed self-assembly as an approach for various functional dendrimers.

Significance

This study demonstrates a unique molecular platform based on the DNA-directed assembly of dendritic

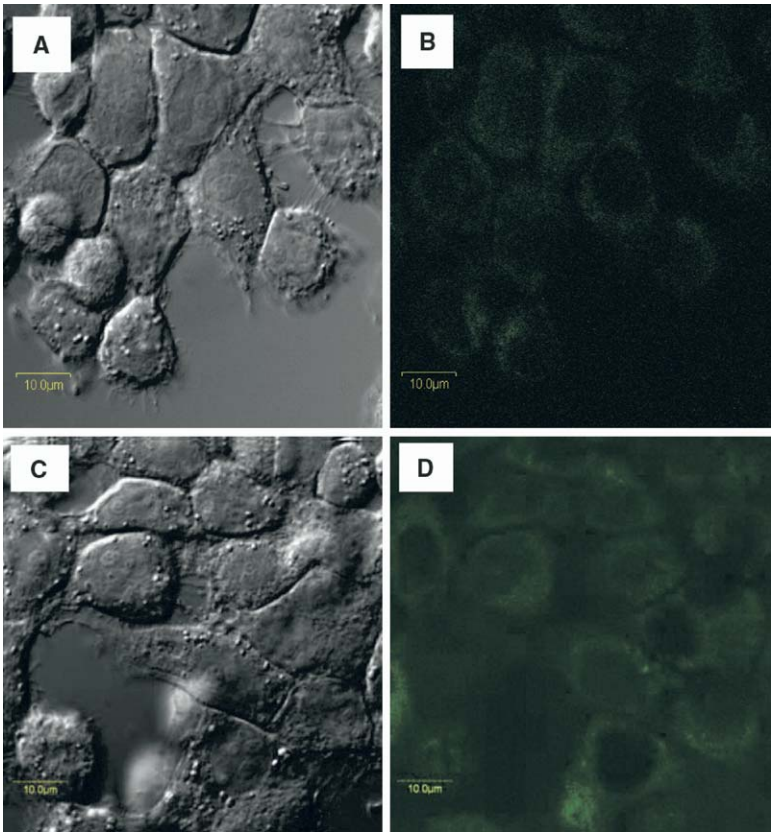

Figure 6. Confocal Microscopic Images of KB Cells Treated with DNA-Linked Cluster of G5-FITC and G5-FA Dendrimers (40 nM)

Left panels show differential interference contrast (DIC) images which correspond to fluorescent images (right panels) of the same slide with KB cells. Compared to the control KB cells in PBS (A and B), the KB cells treated with the DNA-linked functional dendrimer cluster (C and D) show the presence of the FITC signal inside the cells, indicating the binding and internalization of the cluster agent over 1 hr incubation.

polymers for the delivery of different agents to cancer cells. While only nanometers in diameter (the size of proteins), this material allows for the delivery of drugs, genetic materials, and imaging agents to cancer cells through their folate receptors. The unique aspect of this work is that using DNA as a functional linker of dendrimers may allow the combination of different drugs with different targeting and imaging agents in comparison to what can be accomplished with traditional chemistry on a single dendrimer using a limited number of subunits. Using a single dendrimer as a targeted drug delivery platform would require different targeted drugs for varied molecular alterations in each specific cancer cell. This DNA-linked dendrimer nanocluster platform also offers the potential for developing combinatorial therapeutics (different drugs with radiotherapeutics and imaging agents).

Experimental Procedures

Materials

The complementary single-stranded DNA oligonucleotides were synthesized with 5′-phosphate modification and purified by PAGE at Integrated DNA Technologies (Coralville, IA): cON34 (5′-[P]GGCCATATAGGCCTTTTGGCCTATATG GCCGGGGG-3′, MW = 10,585.8 g/mol, extinction coefficient [M^{-1} cm^{-1}] = 318,300, T_m = 71.2°C); sON34 (5′-[P]CCCCGGCCATATAGGCC AAAAGGCCTATAT GGCC-3′, MW = 10,461.7 g/mol, extinction coefficient [M^{-1} cm^{-1}] = 322,500, T_m = 71.2°C). The generation 5 polyamidoamine (PAMAM) dendrimer was synthesized at the Center for Biologic Nanotechnology and analyzed by ^{1}H, ^{13}C NMR, SEC-MALLS, and potentiometric titration and MALDI-TOF mass spectrometry. Fluorescein isothiocyanate (FITC), imidazole hydrochloride, acetic anhydride, triethylamine, anhydrous methanol, and dimethyl sulfoxide (DMSO) were purchased from Aldrich (St. Louis, MO). Folic acid, acetic acid, dimethyl sulfoxide (DMSO), penicillin/streptomycin, fetal bovine calf serum (FBS), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Trypsin-EDTA, Dulbecco's PBS (DPBS), and RPMI 1640 medium (with or without FA) were obtained from GIBCO-BRL (Gaithersburg, MD).

Partial Acetylation of Generation 5 PAMAM Dendrimer

To enhance the solubility of the dendrimer conjugates and reduce nonspecific interaction with cell membranes, the surface primary amines were partially acetylated using acetic anhydride as previously described. Briefly, 82 equivalents of acetic anhydride (0.21 ml, 2.2 mmol; 70% ratio of terminal primary amine of dendrimer) was slowly added to the amine-terminated G5 PAMAM dendrimer (0.7 g, 26.9 μmol) in anhydrous MeOH (70 ml) in the presence of triethylamine (0.38 ml, 2.75 mmol). The mixture was stirred under N_2 atmosphere for 18 hr at room temperature. Excess solvent and reagents were removed by rotary evaporation, followed by extensive dialysis (MWCO = 3.5 kDa) in 0.2 M NaCl and deionized water for 3 days. After lyophilization, the sample was obtained as a white powder (782 mg, 85% yield). ^{1}H NMR (500 MHz, D_2O) δ 3.3 (-NHCH_2), 2.8 (-CH_2), 2.7 (-CH_2NH_2), 2.5 (-CH_2CO), 1.8 (-$NHCH_3$). ^{13}C NMR (500 MHz, D_2O) δ 175.9, 175.2, 174.8, 174.5 (C=O); 51.7, 49.5, 39.6, 39.1, 37.1, 33.0 (-CH_2-); 22.4 (-CH_3). See Supplemental Data for more detailed structural analysis.

Conjugation of FITC to the Partially Acetylated Generation 5 PAMAM Dendrimer

Five molar equivalents of FITC (6.5 mg, 15 μmol) dissolved in DMSO (2 ml) was slowly added to a solution of the partially acetylated dendrimer (100 mg, 3 μmol) in DMSO (8 ml) in a nitrogen atmosphere. After 24 hr, the reaction mixture was concentrated and the solvent was exchanged with PBS. The solution was purified first by gel filtration chromatography (Sephadex G-25M) to remove excess FITC, followed by exhaustive dialysis against PBS (pH 8.0) and deionized water using a 3.5 kDa MWCO membrane for 3 days.

The final product was obtained as an orange powder after lyophilization (90 mg, 84.6% yield). ^{1}H NMR (500 MHz, D_2O) δ 7.9, 7.7 (CH), 7.12 (-CH), 6.6 (-CH), 3.3, 3.1 (-$NHCH_2$), 2.84 (-CH_2), 2.65 (-CH_2NH_2), 2.43 (-CH_2-CO), 1.97 (-NH-CH_3). ^{13}C NMR (500 MHz, D_2O) δ 174.4, 51.7, 49.5, 39.0, 36.9, 32.8, 22.4. MALDI-TOF (m/z): found $[M + H]^+$ 32,571. See Supplemental Data for further details.

Conjugation of FA to the Partially Acetylated Generation 5 PAMAM Dendrimers

Folic acid (MW = 527.53 g/mol) was used to prepare a dendrimer which will function as a targeting unit. The partially acetylated dendrimer (50 mg, 1.69 μmol) was dissolved in DI water (60 ml). The γ-carboxylic acid of the folic acid (5.4 mg, 12.17 μmol) was first activated by EDC (121.3 mg) in DMF/DMSO (27 ml, 3:1, v/v) solution. After 90 min, the folate solution was slowly added to the dendrimer solution and then stirred at room temperature for 2 days. The reaction mixture was purified by gel filtration chromatography (Sephadex G-25M) followed by extensive dialysis in PBS and water for 3 days with seven solvent exchanges, followed by lyophilization (52 mg yellow powder, 80% yield). ^{1}H NMR (500 MHz, D_2O) δ 8.6, 8.0 (-CH), 7.6 (-CH), 6.7 (-CH), 3.75, 3.62, 3.51, 3.3 (-$NHCH_2$), 2.8 (-CH_2), 2.6 (-CH_2NH_2), 2.39 (-CH_2CO), 1.95 (-$NHCH_3$). ^{13}C NMR (500 MHz, D_2O) δ 175.2, 174.8, 174.4, 72.4, 62.8, 51.7, 49.5, 39.1, 37.1, 33.0, 22.3. MALDI-TOF (m/z): found $[M + H]^+$ 32,505, $[M + H]^{2+}$ 16,001. See Supplemental Data for additional detail.

Oligonucleotide Conjugation to the FA- or FITC-Modified Dendrimers

The 5′-phosphate-modified oligonucleotide (cON34; 64 μg, 6.2 nmol) was activated by EDC (10 mg) in 0.1 M imidazole buffer (pH 6.0) solution. The activated oligonucleotide was then slowly added to the FITC-conjugated G5 dendrimer (1 mg, 30 nmol) dissolved in 0.5 M LiCl. While stirring, additional LiCl was added to the solution to make the final LiCl concentration of the reaction mixture (0.5 M). After 18 hr, the reaction mixture was purified with a 10 kDa MWCO regenerated cellulose filtration device (Centricon YM-10; Millipore, Billerica, MA) to remove excess EDC, LiCl, and isourea byproducts and concentrated ten times. In the case of the oligonucleotide conjugation to the FA-modified dendrimer, the 5′-phosphate-modified oligonucleotide (sON34; 65 μg, 6.2 nmol), which is complementary to cON34, was activated by EDC (10 mg) in 3 ml of 0.1 M imidazole buffer solution (pH 6.0). The activated oligonucleotide was then slowly added to the FA-conjugated dendrimer (1 mg, 30 nmol) dissolved in 0.5 M LiCl. After 18 hr, the reaction mixture was purified with Centricon YM-10.

Hybridization of the DNA-Conjugated G5-FITC and G5-FA Dendrimer

Hybridization of the DNA-conjugated functional dendrimers was performed in 10 mM phosphate buffer (pH 7.4), 150 mM NaCl. Each DNA-dendrimer conjugate (11 μM) was mixed at equal volume and diluted in PBS solution (1 ml). Then, the mixture was heated at 90°C for 10 min, followed by cooling to room temperature over 3 hr. The resulting reaction mixture was purified by ultrafiltration using a 100 kDa MWCO membrane (Centricon YM-100) with washes (×3) with PBS (1000 g, 15 min). Because the final DNA-linked dendrimer cluster is expected to have approximately 88 kDa while each functional dendrimer is 33 kDa, the use of the membrane allowed for separation of the unreacted excess dendrimers and unhybridized DNA-dendrimer conjugates from the clustered dendrimers with a yield of recovery of 25%.

General Characterization Methods

UV-vis spectra were obtained in a 0.5 ml quartz cuvette using a Perkin Elmer Lamda 20 spectrophotometer (Boston, MA) at room temperature. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AVANCE DRX 500 MHz spectrometer (Billerica, MA) with samples dissolved in D_2O at a concentration of 2–5 mg/ml.

SEC-MALLS experiments were performed using an Alliance Waters 2690 separation module (Waters Corporation, Milford, MA) equipped with a Waters 2487 UV absorbance detector (Waters), a Wyatt Dawn DSP laser photometer (Wyatt Technology Corporation, Santa Barbara, CA), an Optilab DSP interferometric refractometer (Wyatt Technology), and TosoHaas TSK-Gel Guard PHW 06762, G 2000 PW 05761, G 3000 PW 05762, and G 4000 PW columns (TosoHaas, Grove City, OH). Column temperatures were maintained at 25 ± 0.2°C with a Waters temperature control module. Citric acid buffer (0.1 M) with 0.025% sodium azide in water was used as mobile phase. The pH of the mobile phase was adjusted to 2.74 using NaOH and the flow rate was maintained at 1 ml/min. Sample concentration was approximately 2 mg/ml and an injection volume of 100 μl was used for all samples. M_n and M_w and polydispersity index (PDI) of the polymer conjugates were determined using Astramol software (version 4.7; Wyatt Technology). The instrument calibration constants of the GPC apparatus were determined using polyethylene glycol (PEG) and polyethylene oxide (PEO) standards from American Polymer Standards (Mentor, OH). The standards used were PEG15,000 (M_n = 13,200 g/mol, PDI = 1.1) and PEOX20K (M_n = 20,500 g/mol, PDI = 1.17). Once the instrument calibration constants were determined, the absolute molar masses of dendrimer samples could be evaluated without further calibration.

HPLC analysis was performed using an RP-HPLC system consisting of GOLD 126 solvent module, model 507 autosampler equipped with a 100 μl loop, and a model 166 UV detector (Beckman Coulter, Fullerton, CA). A Jupiter C5 silica-based RP-HPLC column (250 × 4.6 mm, 300 Å) was purchased from Phenomenex (Torrance, CA). Two Phenomenex Widepore C5 guard columns (4 × 3 mm) were also installed ahead of the Jupiter column. The mobile phase was a linear gradient beginning with 100:0 water/acetonitrile (ACN) at a flow rate of 1 ml/min, reaching 70:30 after 40 min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in ACN was used as a counterion to make the dendrimer surfaces neutral. The dendrimer-TFA salt adsorbs strongly to the stationary phase compared to the amine-terminated dendrimer itself. The injection volume in each case was 50 μl with a sample concentration of 1 mg/ml and the detection of eluted samples was performed at 210 nm. The elution profile was analyzed using Beckman's System Gold Nouveau software.

The MALDI-TOF MS were acquired using a Micromass TofSepc-2E calibrated with protein standards (cytochrome C, myoglobin, and trypsinogen), operating in positive ion mode. For each sample, 5 μl of sample solution in methanol was mixed with 5 μl of the matrix solution. The matrix solution was a 10 mg/ml solution of 2,5-dihydroxybenzoic acid (DHBA) in ACN/water (70:30) with 0.1% TFA. One microliter of the sample/matrix mixture was spotted on the MALDI plate and evaporated to dryness.

Cell Cultures and Biological Evaluation

The KB cells were purchased from the American Type Tissue Collection (ATCC, Manassas, VA) and continuously grown in two 24-well plates, one in FA-free media and the other in regular RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FBS, yielding a final FA concentration approximately that of normal human serum at 37°C, 5% CO_2. Approximately 2 × 10^6 cells per well were seeded in 12-well plates the day before the experiments. An hour before initiating an experiment, the cells were rinsed four times with serum-free and FA-deficient RPMI 1640 media. DNA-linked dendrimer cluster was added in the final concentrations of 5–80 nM.

Several control groups were designed to prove that the DNA linker strategy we employed for this cluster agent construction truly plays an important role in targeting the two functional dendrimers in vitro. For example, a consecutive addition of two different samples, (1) G5-FA followed by G5-FITC, and (2) G5-FA-sON34 then G5-FITC-cON34, was performed as follows. After the addition of the first sample, the plate was incubated for 20 min at room temperature. The incubation medium was aspirated from wells, rinsed once, and replaced with fresh medium. Then, the second set of dendrimer samples was added and incubated for 1 hr at 37°C. The medium was aspirated off, rinsed once with PBS, trypsinized using a standard protocol, and resuspended in 0.4 ml Dulbecco's PBS. The quantitative analysis of the DNA-linked functional dendrimers' fluorescence intensity in KB cells was performed with a flow cytometer (Epics XL-MCL, Beckman Coulter). Surface and interior localization of the DNA-linked functional dendrimers were qualitatively determined using an Olympus FluoView 500 laser scanning

confocal microscope (Melville, NY). FITC fluorescence was excited with a 488 nm argon blue laser and emission was measured through a 505–525 barrier filter. Samples were scanned on an Olympus IX-71 inverted microscope using a 60× water immersion objective and magnified three times with FluoView software. The changes in cell fluorescence were expressed in arbitrary units and plotted against the concentration of the DNA-linked functional dendrimers.

Supplemental Data

Supplemental Data are available at http://www.chembiol.com/cgi/content/full/12/1/35/DC1/.

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