Poly(ethylene glycol)-Conjugated PAMAM Dendrimer for Biocompatible, High-Efficiency DNA Delivery

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ABSTRACT: We demonstrate a simple and successful synthetic approach to devise a highly efficient DNA delivery system with low cytotoxicity and low cost. Polyamidoamine (PAMAM) dendrimer is a highly efficient DNA delivery agent, when compared to other chemical transfection reagents. Partially degraded, high-generation dendrimers offer even higher efficiency, presumably due to enhanced flexibility of the otherwise rigid dendrimer chains.[1] We hypothesized that chemical modification of low generation dendrimer with biocompatible poly(ethylene glycol) (PEG) chains would create a conjugate of PAMAM core with flexible PEG chains, which mimics the fractured high-generation dendrimer and produces high transfection efficiency. Generation 5 PAMAM was modified with 3400 molecular weight PEG. The novel conjugate produced a 20-fold increase in transfection efficiency compared with partially degraded dendrimer controls. The cytotoxicity of PEGylated dendrimers was very low. This extremely efficient, highly biocompatible, low-cost DNA delivery system can be readily used in basic research laboratories and may find future clinical applications.

Introduction

DNA delivery is a key technique in basic biological research and gene therapy. Viral-mediated DNA delivery (infection) has played a major role in gene therapy. Unfortunately, initial enthusiasm has been tempered by growing concerns regarding the toxicity and immunogenicity associated with viral vectors. Synthetic DNA delivery systems (transfection), on the other hand, provide potential alternatives for gene therapy and DNA vaccination. The major drawback of current transfection technology is low efficiency compared to infection techniques, although new materials are being developed that promise to overcome some of the major barriers to DNA delivery.[2]

One such material is polyamidoamine dendrimer (PAMAM), which has been extensively studied for its role in transfection.[3,4] PAMAM dendrimers are nanoscopic spherical macromolecules composed of polyamidoamino units with repeating dendritic branching. Because of the presence of protonated primary amine groups on the surface, these highly branched dendrimers possess a high, positive charge density that is responsible for both the ionic condensation of DNA and binding to the cell surface.[4] Protonated residues may also provide endosomal buffering and thus protect DNA from lysosomal degradation.[5] High generation PAMAM and fractured PAMAM ("SuperFect") have been shown to increase transfection efficiency 10−100-fold over lipid-based reagents.[1,6]

Other modified polymers have also been tested for enhancement of DNA delivery. Poly(ethylene glycol)-based copolymers have been used extensively in protein delivery systems because of their high water-solubility, nonimmunogenicity, and biocompatibility.[7,8] PEGylation has also been successfully applied to produce various DNA delivery agents. For example, lactose-PEG-grafted poly-L-lysine polymers are efficient DNA carriers;[9] a barbell-like triblock copolymer, poly(L-lysine) dendrimer-*block*-PEG-*block*-poly(L-lysine) dendrimer (PLLD−PEG−PLLD), exhibits low cytotoxicity when complexed with DNA;[10] and a water-soluble block copolymer, PEG-*block*-poly(L-lysine), complexes with DNA and provides resistance to DNase I attack.[11,12] Recently, a water-soluble dendrimer−PEG conjugate that may have potential as a drug carrier was also described.[13]

Despite intense efforts to increase transfection efficiency via the nonviral route,[2] biocompatible, low-cost, efficient transfection reagents are still not available. We hypothesized that low generation PAMAM conjugated with PEG would mimic some of the essential properties of the fractured high generation PAMAM (i.e., SuperFect), with the PEG chains serving the same role as the unfractured PAMAM chains. This conjugate would have several potential advantages. PEGylation would add flexibility and accommodate the DNA helices without creating strong electrostatic interactions. Biocompatibility of the molecules would improve due to the reduction in generation of PAMAM and addition of biocompatible PEG branches. In addition, intracellular release of DNA molecules is expected to be more efficient, and potentially adjustable, due to the presence of a controlled number of surface amino groups and controlled length of the PEG arms.

Here, we report on the synthesis and characterization of a PEG−PAMAM conjugate that increases transfection up to 20-fold compared with the partially degraded dendrimer SuperFect, which is one of the best transfection reagents currently available. This PEG−PAMAM conjugate is easy to prepare, nontoxic to cells, and low-cost and may be used to increase transfection efficiency in cell culture. Furthermore, our model system—dendritic polymer modified with grafted linear polymer chains—may serve as a starting point for rational design of synthetic DNA delivery systems in the future.

Experimental Section

Materials. PAMAM (Starburst) was purchased from Dendritech (Midland, MI) or Sigma (St. Louis, MO); NHS−

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PEG3400−NH−tBoc was obtained from Shearwater Polymers (Huntsville, AL). SuperFect transfection reagents were purchased from Qiagen, Inc. (Valencia, CA). Cell lines and culture media were acquired from ATCC (Manassas, VA). All other chemicals were obtained from Sigma.

Synthetic Procedure. The condensation reaction between PAMAM and the activated ester of HOOC−PEG3400−NH−tBoc was carried out in a borate buffer (pH = 8.0) based on our previous experience.[7,8] The starting materials were the following.

1. PAMAM ($g = 3$, $f = 32$ amino groups, MW = 6909, or $g = 4$, $f = 64$ amino groups, MW = 14 215, or $g = 5$, $f = 128$ amino groups, MW = 28826).

2. NHS−PEG3400−NH−tBoc (MW = 3350 by MALDI)

Four different PEG−PAMAM conjugates were produced.

PEG−PAMAM-3: PAMAM ($g = 3$), 3.24 mM, 22.4 mg, and NHS−PEG, 11.6 mM, 38.9 mg.

PEG−PAMAM-4: PAMAM ($g = 4$), 1.62 mM, 23.0 mg, and NHS−PEG, 11.6 mM, 38.9 mg.

PEG−PAMAM-5: PAMAM ($g = 5$), 0.81 mM, 23.3 mg, and NHS−PEG, 11.6 mM, 38.9 mg.

PEG−PAMAM-5S: PAMAM ($g = 5$), 0.08 mM, 2.4 mg, and NHS−PEG, 11.5 mM, 38.4 mg.

The solvent (methanol) was vacuum-evaporated from PAMAM prior to reaction. PAMAM mass, reported above, was the actual mass after solvent evaporation. The two starting compounds were dissolved in borate buffer (pH = 8.0) and incubated at room temperature for 24 h with shaking. Crude reaction mixtures of conjugates were dialyzed against distilled water using dialysis tubing with MWCO = 10 000 for 24 h before lyophilizing.

Physical Characterization of PEG−PAMAM. Fourier transform infrared (FT-IR) spectra were collected with a Mattson 2020 Galaxy series instrument at a resolution of 3.0 cm^{-1} on powder KBr samples. Mass spectrometry of PEG−PAMAM-5 and −5S conjugates was performed with both low-resolution electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Proton NMR was done in $CDCl_3$ using a Varian Inova-400. Ninhydrin tests were run to quantify primary amines using the following procedure. A calibration curve was constructed for PAMAM ($g = 5$), which was dissolved at 2 mg/mL in ethanol. A total of 10−80 μL of PAMAM solution was combined with 750 μL of ninhydrin (0.4 wt % in ethanol) and diluted to 1 mL in ethanol. Solutions were heated for 4 min to about 90% evaporation. Each cooled sample was diluted to 5 mL with ethanol, and the UV absorbance at 590 nm was measured. PEG−PAMAM conjugates were dissolved in ethanol (2 mg/mL), heated with ninhydrin, and measured at 590 nm to determine the number of reactive primary amines, relative to the PAMAM standards.

Complexation with DNA. DNA was complexed with the conjugates by mixing in potassium phosphate buffered water at room temperature. Depending on the experimental design, the ratio of DNA to conjugate, the pH of the buffer solution used, and the incubation time were varied.

Electrophoresis. SDS−PAGE was performed on 12% and 4−20% Tris-HCl gels at room temperature for 1 h at 200 V using a Mini-Protein II cell (BioRad, Hercules, CA) system. Bands were visualized by silver staining. Agarose gel electrophoresis was carried out in a mini unit (Embi Tec, San Diego, CA) at 100 constant volts. A double-stranded DNA-specific fluorescent dye (SYBR, Molecular Probes, Eugene, OR) was used to detect DNA bands.

Cell Culture. Chinese hamster ovarian (CHO) cells were cultured in F12K medium. All media preparations contained 10% fetal calf serum and 1% penicillin/streptomycin. Cells were cultured at 37 °C in 5% CO_2.

Transfection. Transfections were carried out according to established protocols for fractured dendrimer (e.g., for SuperFect, Qiagen Inc., Valencia, CA). Briefly, plasmid DNA coding for a reporter protein β-galactosidase (β-gal) was first complexed with dendrimer or conjugate at room temperature before addition to the cells. After incubation at 37 °C in a 5% CO_2 atmosphere for 2 h, medium containing the mixtures was gently removed and fresh growth medium was added. To assess the transfection efficiencies (24 h post transfection), cells were first lysed using M-PER buffer (Pierce, Rockford, IL), and enzyme activities were determined using a β-gal assay kit (Promega). A standard curve was constructed for each experiment using dilutions of purified β-gal protein. The β-gal activities from experimental samples were determined by comparison to the standard curve.

Cytotoxicity Assays. Cytotoxicity was assessed by total protein assays and MTS assays ([3-(4,5-dimethylthiazol-2-yl)-5-(3-caroxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. In the total protein assays, the change in cell number upon exposure to test compounds was quantified using total protein content. Briefly, cells cultured on a 6-well plate were incubated with PEGylated PAMAM or PAMAM alone per experimental design. Cells were lysed 24 h later using 500 μL of M-PER buffer (Pierce, Rockford, IL). Protein concentrations were obtained by routine Coomassie Blue assay reagents using bovine serum albumins as standards (Pierce). Nontreated cells and SuperFect-treated cells were used as controls. In the MTS assays, the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was used according to manufacturer's protocols (Promega, Madison, WI). Briefly, cells were cultured with or without PEGylated PAMAM. MTS reagents were added and bioreduced by metabolic active cells (i.e., live cells) into a colored formazan product that could be quantified by absorbance at 490 nm. Nontreated cells were used as controls. In both cases, the cytotoxicity results were expressed as percentages of total cellular protein or total absorbance compared to nontreated cells.

Calculation of Expected PEG−PAMAM-5 Polydispersity. A MALDI spectrum was acquired to determine the molecular weight distribution of the NHS−PEG3400−NH−tBoc. The largest 27 peaks were included in calculating the mole fraction of each species based on its peak height. The average molecular weight was determined to be 3350. PAMAM was assumed to be monodisperse with a molecular weight of 28826. PEG chains were randomly attributed to the PAMAM molecules until an average of 13.7 PEG per PAMAM was achieved. These calculations gave the expected fractions of PEG−PAMAM-5 conjugates with X PEG chains. For each discrete PEG:PAMAM (e.g., 14:1), the polydispersity of the PEG3400 was then taken into account. The 27 PEG weights were first assumed to be equally probable, and the expected distribution of molecular weights in discrete $-CH_2-CH_2-O-$ steps from PAMAM + NPEG1 to PAMAM + NPEG27 was determined, where N is the number of PEG per PAMAM. To account for the unequal distribution of PEG molecular weights, each probability was multiplied by the MALDI PEG mole fraction associated with the conjugate's average PEG molecular weight. The resulting values were then normalized for each PEG:PAMAM ratio and multiplied by the mole fraction for that PEG:PAMAM ratio to give the expected mole fraction for each discrete molecular weight.

Results

Synthesis and Characterization of PEG−PAMAM. PAMAM (generation 3) was conjugated to PEG first to optimize reaction conditions for PEGylation. SDS−PAGE with silver staining (Figure 1a) demonstrated the formation of high molecular weight products. The main band was found at a position corresponding to the 66K protein marker, which would correspond to PEG−PAMAM-3 with conjugation of seventeen of the 32 PAMAM amino groups (although we realize that the conjugates probably migrate at different rates than protein markers of the same molecular weight). Unmodified PAMAM was not detected on the gel under these conditions (data not shown). FT-IR spectra also confirmed the formation of conjugates by the presence of a new amide bond (1672 cm^{-1}, data not shown). PEG−PAMAM-3 was used to complex with reporter (β-gal) plasmid DNA before transfecting CHO cells. Low

Table 1. Physical Properties of PAMAM and PEG−PAMAM Conjugates

version	av[a] no. of PEG chains	PAMAM[b] diameter, nm	av conjugate MW	conjugate[c] vol, nm^3	conjugate[d] diameter, nm	av primary amines	shielded[e] primary amines
PAMAM-3	0.0	3.6	6909	24.4	3.6	32.0	0
PEG−PAMAM-3	3.4	3.6	17 976	156.3	6.7	28.6	ND
PAMAM-4	0.0	4.5	14 215	47.7	4.5	64.0	0
PEG−PAMAM-4	6.9	4.5	36 675	315.4	8.4	57.1	ND
PAMAM-5	0.0	5.4	28 826	82.4	5.4	128.0	0
PEG−PAMAM-5	13.7	5.4	73 420	613.9	10.5	114.3	∼34
PEG−PAMAM-5S	31	5.4	129 731	1285.0	13.5	97	97

[a] Determined by NMR spectroscopy. [b] Theoretical diameter reported by Dendritech, Inc. [c] Calculated from PAMAM diameter + PEG diameter of 4.2 nm for PEG3400. [d] From conjugate volume assuming a sphere. [e] As not detected by ninhydrin.

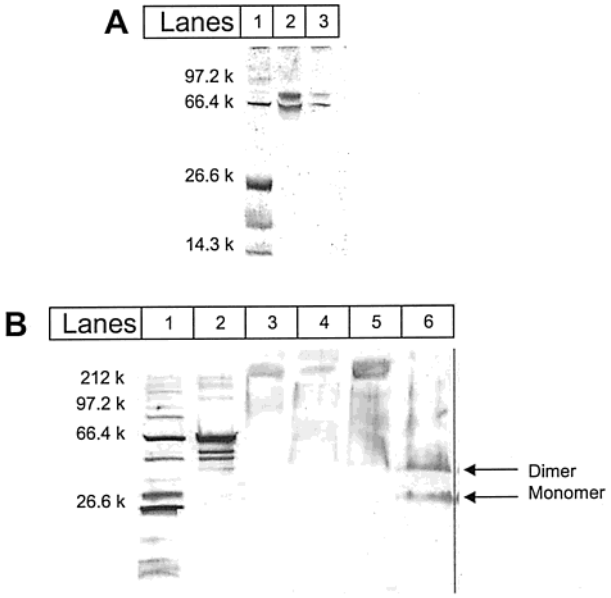

Figure 1. SDS−PAGE of PEG−PAMAM conjugates. (A) PEG−PAMAM-3 conjugates separated on 12% Tris-HCl gel. Lane 1: protein molecular weight marker. Lanes 2 and 3: PEG−PAMAM-3 conjugates, 180 and 120 μg, respectively. (B) PEG−PAMAM-5 conjugates separated on 4−20% gradient gel. Lane 1: protein molecular weight marker. Lane 2: bovine serum protein marker (0.5 μg). Lanes 3, 4, and 5: PEG−PAMAM-5 conjugates, 38, 57, and 96 μg, respectively. Lane 6: Non-PEGylated PAMAM (g = 5), 37 μg.

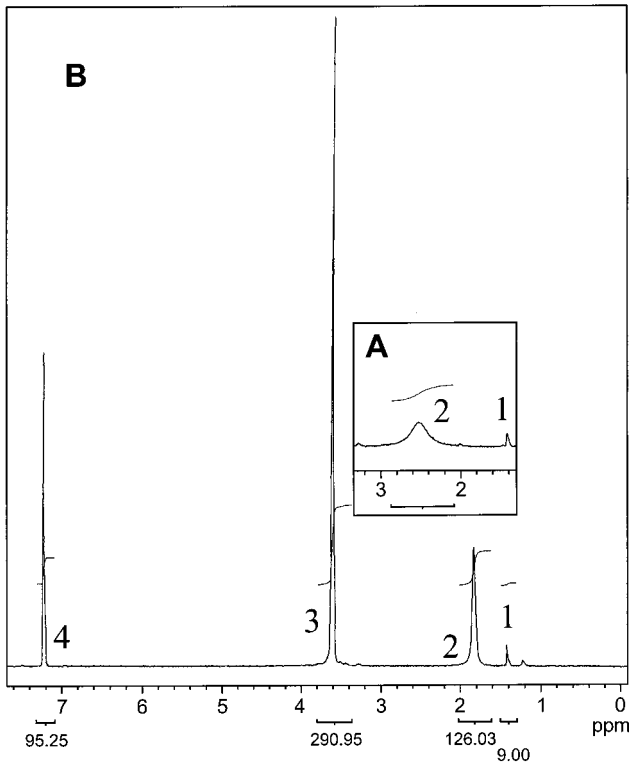

Figure 2. Proton NMR spectra. (A) PEG−PAMAM-5. Peak 1: tBoc. Peak 2: PAMAM $-CH_2-$. (B) PEG−PAMAM-5S. Peak 1: tBoc. Peak 2: PAMAM $-CH_2-$. Peak 3: PEG $-CH_2-$. Peak 4: $CDCl_3$.

β-gal activities were detected, probably due to the small size of the PEG−PAMAM-3 molecules (d < 10 nm, see Table 1).

To increase conjugate size, PAMAM (g = 5) was PEGylated, using the same conditions worked out for PAMAM (g = 3). The cost of PAMAM (g = 5), although a little higher than PAMAM (g = 3), is still 10 to 250 times lower than PAMAM (g > 5), which are used as the basis for other transfection reagents such as SuperFect. SDS−PAGE of PEG−PAMAM-5 (Figure 1b) demonstrated the formation of high molecular weight conjugates that could not enter the gel (lanes 3, 4, and 5). No bands were found below 60K of the marker protein, implying that no uncoupled dendrimers were present. FT-IR spectra of the starting compounds, NHS−PEG3400−NH−tBoc and PAMAM (g = 5) along with the dialyzed, lyophilized, conjugated product, showed the formation of new amide bonds (at 1672 cm^{-1}), consistent with successful condensation. A peak at 1120 cm^{-1} resulted from ether bonds of PEG fragments of the conjugates, although some traces of non-bound NHS−PEG3400−NH−tBoc could not be excluded. The tBoc ester peak appeared at 1724 cm^{-1}. Since no free PAMAM (g = 5) was detected in the reaction mixture on the SDS−PAGE (Figure 1b), the amide bond peaks at 1566 and 1660 cm^{-1} probably represent the dendrimer portion of the conjugate.

In addition to the PEG−PAMAM-5 conjugate described above, PEG−PAMAM-5S, a "saturated" conjugate, was synthesized under identical conditions but with 144 molecules of NHS−PEG3400−NH−tBoc available per molecule of PAMAM. Barring steric limitations on reaction, all 128 primary amines were expected to be PEGylated to form PEG−PAMAM-5S. Nuclear magnetic resonance (NMR) spectroscopy, a ninhydrin test to assay primary amines, and mass spectrometry (MS) were run to characterize these two conjugate versions. A MALDI mass spectrum of NHS−PEG3400−NH−tBoc indicated that the average molecular weight of this reagent was 3350. For PEG−PAMAM-5 and −5S, no conjugate species were detected by MALDI or ESI mass spectrometry. In $CDCl_3$, methylene protons from PEG3400 were found at 3.61 ppm by NMR (Figure 2). For PEG−PAMAM-5, PAMAM methylene protons were found as a broad peak at 2.7 ppm (Figure 2a); the PAMAM peak for PEG−PAMAM-5S was much sharper

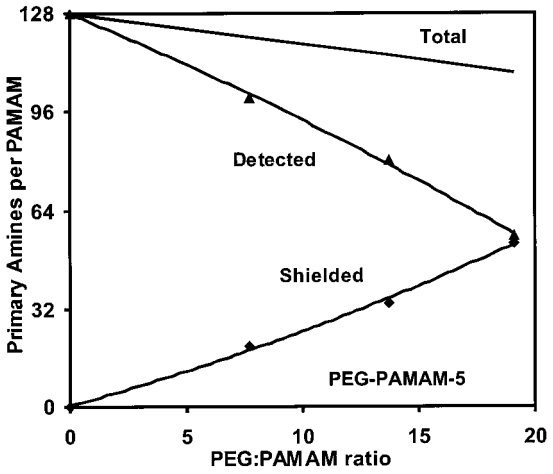

Figure 3. Primary amine accessibility to ninhydrin in PEG−PAMAM conjugates.

and shifted to 1.83 ppm (Figure 2b). A peak at 1.42 ppm in each spectrum indicated that the tBoc group remained on the amino terminus of the PEG. The integrated areas of NMR peaks were used to quantify the number of PEG chains per PAMAM with the assumption of 2032 methylene protons per PAMAM and 278 per PEG. PEG−PAMAM-5 had a PEG/PAMAM proton ratio of 1.87, implying an average of 13.7 PEG chains per PAMAM, a reaction yield of 95.7% based on PEG:PAMAM ratio, and an average molecular weight of 73 420 g/mol.

The presence of free primary amino groups was assessed by a ninhydrin test. Both PEG−PAMAM-5 and −5S versions were compared to PAMAM ($g = 5$) at 590 nm. On a weight/weight basis, the absorbance ratios were 0.2450 and 0.0003, respectively. These absorbance ratios indicate the detection of 80 of the 114 PEG−PAMAM-5 free primary amines and ∼0 PEG−PAMAM-5S free primary amines (Table 1). The ninhydrin test therefore indicated that there are "shielded" primary amines on PEG−PAMAM-5, which do not react with the ninhydrin, and that no primary amines are accessible to ninhydrin in PEG−PAMAM-5S. We wished to determine the actual number of PEG chains attached per PAMAM in "saturated" PEG−PAMAM-5S. Assuming a 95.7% PEG:PAMAM yield, conjugates with PEG:PAMAM ratios of 7.7 and 19.1 were synthesized in addition to 13.7. Ninhydrin tests were performed on each of these conjugates, and the results are shown in Figure 3. Extrapolating the "shielded" primary amine line to its intersection with the "total" line indicates that all free primary amine will be shielded at a PEG:PAMAM ratio of 31. Therefore, we assumed that PEG−PAMAM-5S has a molecular weight of 130 000 (Table 1). We attempted to synthesize conjugates with ratios between 19 and 31, but ninhydrin results indicated that the yields were significantly lower than 95.7% for these higher ratios.

Complexation of PEG−PAMAM and DNA. The formation of DNA and PEG−PAMAM-5 complexes was investigated using DNA mobility retardation assays on agarose gels via electrophoresis (Figure 4). A fixed amount of DNA (1.5 μg) was incubated with PEG−PAMAM-5 at various ratios under the same conditions used for transfection. No free DNA bands were observed after SYBR staining (Figure 4a, lanes 3−6). The detection limit of SYBR staining is 20 picograms per band, which indicates that virtually all DNA (>99.999%) was

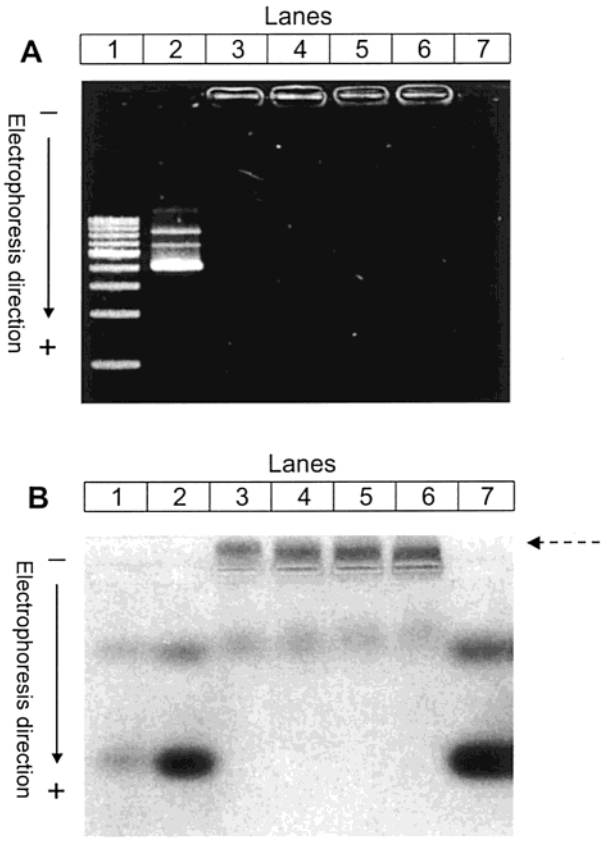

Figure 4. Complexation of DNA and PEG−PAMAM. Agarose electrophoretic mobility retardation assays were carried at 100 constant volts. (A) Electrophoresis picture taken under an UV illumination after the gel was stained with DNA-specific fluorodye SYBR. Lane 1: DNA marker (1kb ladder). Lane 2: pure plasmid DNA (pCMV−Lac, 1.5 μg). Lane 3, 4, 5, and 6: 1.5 μg of pCMV−Lac DNA complexed with 0.46, 0.92, 1.4, and 2.3 mg of PEG−PAMAM-5, respectively. Lane 7: negative control (loading dye only). (B) Electrophoresis picture taken under non-UV, bright-light illumination. Only loading dyes are visible. Lane assignments were the same as in part A.

complexed with PEG−PAMAM-5. Interestingly, we observed that one of the loading dyes in the samples, Bromophenol Blue, was electrophoresed to the opposite direction as DNA (Figure 4b, lanes 3−6, dashed arrow) only in the presence of PEG−PAMAM-5, indicating that the conjugate was able to bind the usually negatively charged dye and reverse the electrophoresis pattern of the dye. These results suggest that DNA was completely complexed with PEG−PAMAM-5 under transfection conditions, and that the overall charge of the complex was positive, which is consistent with most cationic transfection mechanisms.

Highly Efficient Transfection by PEGylated Fifth Generation PAMAM. The transfection potential of PEGylated PAMAM was tested using a procedure optimized for SuperFect (positive control). Two micrograms of reporter DNA was complexed with 0.5−2.3 mg of PAMAM or PEG−PAMAM. The results indicated that the transfection efficiency of PEG−PAMAM-5 (Figure 5, filled circles) was as much as 20-fold higher than SuperFect; non-PEGylated PAMAM ($g = 5$) was completely inactive (Figure 5, open circles). PEG−PAMAM-3 and -4 were also unable to deliver DNA efficiently (data not shown). The increase in transfection efficiency depended not only on the PEGylation, but also the size of the PAMAM and the ratio of PEGylated PAMAM to DNA (Figure 5), suggesting that the complexation of

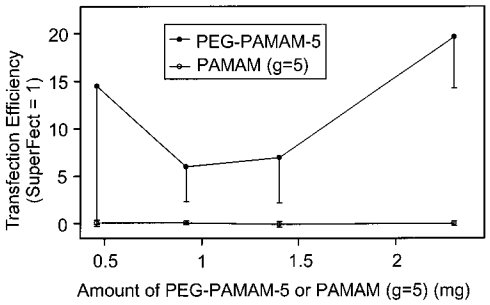

Figure 5. Transfection efficiencies. Two micrograms of reporter DNA was used throughout all the experimental samples except the negative controls where no DNA was. Various amount of PAMAM ($g = 5$) and PEG−PAMAM-5 were tested for their abilities to deliver DNA. Results of transfection were expressed as β-gal activity (mU) normalized to total protein (mg). Fold increase (or decrease) relative to positive controls (SuperFect) under the same conditions was presented as transfection efficiencies. This treatment eliminated the variations commonly seen in transfections. Care was taken to make sure that the presented results were obtained by normalizing to comparable protein contents (protein concentrations were at least 1 mg/mL with no more than 50% of variation among different conditions) and by comparing to successful SuperFect positive controls (β-gal activities were at least 100 mU/mg of total proteins). Filled circles: PEG−PAMAM-5. Open circles: non-PEGylated PAMAM ($g = 5$). Error bars represent the standard deviations calculated from quadruplicates.

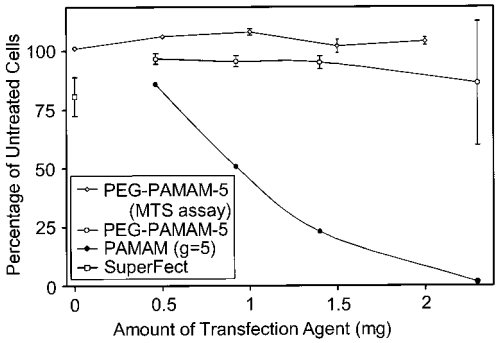

Figure 6. Cytotoxicity assays by total protein content and cell metabolic functions. SuperFect only (30 μg, square, TPC) served as a positive control. Untreated samples were assigned 100%. The results were presented as the percentage of untreated cells. Filled circles: PEG−PAMAM-5 (TPC). Open circles: non-PEGylated PAMAM ($g = 5$) (TPC). Diamonds: PEG−PAMAM-5 by cell metabolic functions. Error bars represent standard deviations calculated from three independent experiments.

PEGylated PAMAM with DNA is one of the keys to enhanced DNA delivery. This result is consistent with previously reported cases of other transfection polymers.[4,9,14]

Cytotoxicity of PEGylated PAMAM. PEGylation is generally viewed as an important step to reduce cytotoxicity and immunogenicity both in vitro and in vivo. We tested the cytotoxicity of PEG−PAMAM-5 in cell culture, compared to non-PEGylated PAMAM and SuperFect. Cytotoxicity of PEG−PAMAM-5 (Figure 6, filled circles) was substantially less than that of non-PEGylated PAMAM (Figure 6, open circles) and was similar to that of SuperFect (Figure 6, square), which has been viewed as one of the least toxic transfection reagents. Similar results were observed using an MTS cytotoxicity assay that quantified the number of metabolically active cells after treatment with PEGylated PAMAM (Figure 6, diamonds).

Discussion

We chose PAMAM dendrimers as a starting point in the design of a high-efficiency, low-cost, nontoxic transfection reagent. The rationale was 4-fold. First, PAMAM dendrimer is a nanoscopic polymer with regular dendritic branching and symmetry. Unlike other polycationic transfection reagents such as poly(L-lysine), the size and architecture of the dendrimer are well-defined.[4] Second, the presence of ordered and protonated primary amine groups on the spherical surface provides easy and equal access for modification. Third, unlike lipid- or protein-based DNA delivery systems, where blood clearance and immunogenicity are concerns, dendrimers appear to have low cytotoxicity both in vitro and in vivo.[15] Fourth, partially fractured, high generation PAMAM dendrimer is arguably one of the best transfection reagents currently known and is available to serve as a positive control to screen for higher efficiency. Therefore, PAMAM dendrimers appeared to be ideal candidates for use as a platform for the rational design of synthetic DNA delivery systems.

In specific, we wanted to create flexibility within the dendrimer conjugates, to control the size of the complexes, to increase the biocompatibility of the reagents, and to reduce the cost of transfection. We chose a low generation dendrimer ($g = 5$) for several reasons. PAMAM Starburst dendrimers are synthesized from a central core by a stepwise process that doubles the number of reactive termini after each generation cycle;[16] for higher generations, more steps are involved, and hence it is more difficult to control the synthesis. Higher generation PAMAM is therefore much more costly (higher generation PAMAM in the form of SuperFect costs 1500-fold more than lower generation PAMAM). In addition, several lines of evidence indicate that higher generation leads to higher toxicity to cells.[15,17] The biocompatibility of our system is further enhanced by modification with PEG, a widely used polymer in drug delivery systems.[18] We chose NHS−PEG3400−NH−tBoc as the modifier because (a) PEGylation is well documented to increase biocompatibility, (b) PEG3400 modifier has a well-defined length, (c) the reaction condensation is relatively simple, (d) the reagents are inexpensive, and (e) we have experience with PEGylation based on our previous work with proteins.[7,8]

The initial conjugate (PEG−PAMAM-5) contained an average of 14 PEG chains per PAMAM ($g = 5$) and produced 15−20 times more transfection efficiency than SuperFect, although significantly greater amounts of this PEG−PAMAM-5 were required to achieve the effect. The standard protocol for SuperFect uses 15 mg per mg of DNA compared to the 250−1200 mg of PEG−PAMAM-5 used in our transfections. This factor does reduce the cost-effectiveness of PEG−PAMAM-5, but such large amounts appeared to have no negative effects on cells. Ninhydrin tests indicated that conjugated PEG is able to prevent uncharged species from interacting with a significant number of primary amino groups on the PAMAM surface. In fact, the 14 PEG chains on PEG−PAMAM-5 were able to shield more than 25% (34 of 114) of the primary amino groups from reaction with ninhydrin in ethanol.

Although physical characterization of the PEG−PAMAM-5 was difficult, we believe that NMR spectroscopy and ninhydrin reactivity together provide a good picture of the conjugate structure. Neither MALDI nor ESI mass spectrometry was able to detect any high

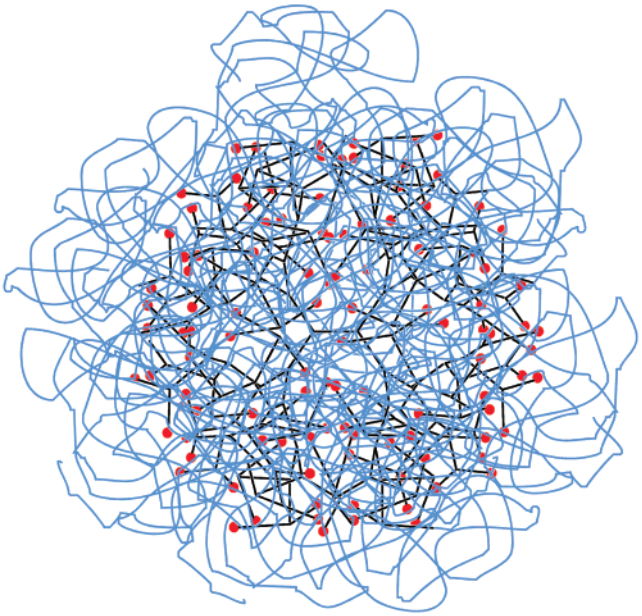

Figure 7. Schematic diagram of a PEG−PAMAM-5 molecule with 14 PEG chains. The PAMAM core is black, and PEG3400 chains are blue. Red circles indicate primary amine sites on the PAMAM.

molecular weight species. This is likely a result of the combination of the high molecular weight of the compounds, the polydispersity of the PEG, and the dispersity in the number of PEG chains per PAMAM. Using the NHS−PEG3400−NH−tBoc molecular weight distribution determined by MALDI, and assuming that the PAMAM sites are filled randomly until 13.7 PEG chains per PAMAM is achieved, we calculated that the highest probability molecular weight species of PEG−PAMAM-5 will represent less than half of a mole percent of the sample (see Experimental Section). Bulk detection of methylene groups by NMR and primary amino groups with ninhydrin, however, indicates the degree of PAMAM modification and the degree of shielding provided by PEG chains for PEG−PAMAM-5. Figure 7 shows a schematic PEG−PAMAM-5 conjugate with 14 PEG3400 chains attached. The scaling between PEG and PAMAM in this diagram and calculations in Table 1 assume a hydrodynamic diameter of 4.2 nm for PEG[19] and a diameter of 5.4 nm for PAMAM ($g = 5$),[20] which are reasonable assumptions given previous measurements on these compounds.

With only 10% of the amino groups (14 of 128) modified with PEG in PEG−PAMAM-5, the dangling PEG chains should have a great deal of flexibility in water, and a significant amount of PEG-shielded surface area should be available on the exterior of the internal PAMAM dendrimer. We believe that the PEG-shielded PAMAM primary amines become charged in water and attract the negatively charged DNA to create PEG−PAMAM-5-DNA complexes. The PEG chains provide steric stabilization to the primary PAMAM−DNA electrostatic association, and this stabilization is a function of PEG chain density. Terminal t-Boc groups may also play a stabilizing role and aid in cell membrane crossing. We also propose that the number and ratio of shielded to unshielded PAMAM primary amines determines complex size and net charge of the complex. Charge separation created by the presence of the PEG chains will ease DNA dissociation once the complex has entered the cell. The optimal PEG−PAMAM transfection conjugate will have the best balance of these complex shielding and chain density factors and will be determined by transfecting with conjugates of different PEG:PAMAM ratios.

We have presented a novel DNA delivery system based on rational modification of PAMAM Starburst dendrimers. This system is highly efficient with low toxicity and low cost. There are three important attributes of this system. It is (1) produced from a low generation of PAMAM ($g = 5$), (2) employing biocompatible modifiers (PEG) and (3) creating shielded PAMAM amines. Though the mechanisms of this highly efficient DNA delivery system are not fully understood and are currently under investigation, we believe that stabilization created by PEGylation is important in binding and delivering DNA. The efficiency of dendrimer-mediated transfection is influenced by amino content, net charge, molecular weight, and polymer flexibility.[1] Recently, Bielinska argued that the transfection activity of DNA−dendrimer complexes is probably due to the soluble, low-density rather than nonsoluble, high-density DNA−dendrimer complexes.[4] Szoka and colleagues degraded PAMAM dendrimers and achieved much higher transfection efficiency, pointing to the important role of polymer flexibility.[1] The addition of the PEG chains may serve a similar role: enhancing the flexibility of PEGylated dendrimers. Finally, due to low-density charges of PEG molecules, DNA is more likely to escape from the complexes once inside cells.

The only drawback of the current PEG−PAMAM-5 preparation as a transfection agent for DNA delivery is the high conjugate:DNA ratio requirement for high transfection efficiency. While others have shown that dendrimer−DNA charge ratios (P/N ratio of PAMAM primary amines to DNA phosphates) in the range 20−50 produce optimal transfection,[4] our results were for P/N ratios in the range 175−825. Even if only "shielded" amino groups, as measured by a ninhydrin test are counted, the dendrimer−DNA ratios are still 50−250. Either a different mechanism is at work for transfection with PEG−PAMAM, or the system is not optimized. We believe that the PEG−PAMAM-5 described here does not possess the optimal balance of PEG and PAMAM, and currently we are investigating other degrees of modification in order to find one that will transfect as efficiently at lower conjugate-to-DNA ratios. For the PEG−PAMAM-5 reaction conditions, about 31 PEG chains can be attached per PAMAM, before attached PEG chains block further attachments. Since PEG−PAMAM-5 was found to have 13.7 PEG chains per conjugate, it is on average 44% "saturated." We believe that slightly increasing the amount of PEG will create a significant increase in "shielded" primary amines, which will reduce the conjugate-to-DNA ratio without compromising transfection efficiency.

Conclusion

We demonstrated a simple and successful synthetic approach to devise a highly efficient DNA delivery system with low cytotoxicity and low cost. This system can be readily used in basic research laboratories and may be modified and applied in future clinical usage. Furthermore, similar approaches could be adopted with other DNA delivery agents, which may create new opportunities for using DNA as a drug via a synthetic delivery route.

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