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Synthesis and Screening of a Small Library of Proline-Based Biodendrimers for Use as Delivery Agents

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Abstract: *A small library of defined peptide dendrimers based on polyproline sequences was designed to demonstrate the feasibility of generating a new type of polymeric agent for therapeutic use. Structural modifications to dendrimer surfaces further enriched the diversity of the library. Data show that the prolinerich dendrimers can be internalized in human epithelial (HeLa) cells, demonstrating the importance of the dendrimeric motif. The promising results described herein suggest that controlled modification of the dendrimer surface should eventually yield proline dendrimers with therapeutic potential.* © 2005 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 80: 800–814, 2005

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INTRODUCTION

Advances in polymer and analytical chemistry have contributed to the development of new materials for industrial and medical applications.[1] Linear polymers have been superseded with more elaborate polymeric structures such as multivalent,[2] branched,[3] graft,[4] dendronized,[5] block,[6] star,[7] and dendrimeric[8,9] motifs. These new polymer classes offer greater definition, richer composition, and higher surface multivalency than linear polymers and allow the creation of special three-dimensional architectures. Dendrimers are highly branched polymers of defined three-dimensional size, shape, and topology built from a series of branches or building blocks around a core. An elevated number of surface end groups are obtained upon each round of outward expansion, or generation, of a dendrimer.[10] Dendrimers have been used in several applications, for example, as artificial enzymes, biocompatible surfactants, and in biomedicine.[11] A wide variety of dendrimers exist, including poly(amidoamine) (PAMAM),[12] poly(L-lysine) (or MAP),[13] poly(propyleneimine) (PPI),[14] and polyester.[15] PAMAM (Starbust™, Aldrich Chemical Company and Dendritech) and PPI (Astramol™, Aldrich and DSM Fine Chemicals) are commercially available.

Analysis of dendrimer architecture reveals two key features that could contribute to a versatile carrier for drug,[16,17] gene,[18–20] or vaccine[21] delivery systems: internal cavities capable of entrapping drugs and a large number of end groups that could lead to covalent or noncovalent attachment of multiple bioactive molecules. Moreover, the supramolecular systems that dendrimers can form, such as dispersions, vesicles, nanoparticles, films, or gels[22] (depending on the conditions) suggest a wide range of possibilities for the delivery. In a broader sense, dendrimers can be considered to be novel polymeric nanomedicines.[23]

Peptide dendrimers based on polyproline helices can be considered biodendrimers since they are built from natural metabolites and are water-soluble polymers, which is decisive for delivery systems.[24] Furthermore, proline-rich sequences are present in peptides capable of crossing cell membranes, a crucial property for delivery of cargo into cells.[25–27] Proline is also a component of collagen,[28,29] in which it is found in GlyXaaYaa triplets such as GlyProHyp. Thus, proline dendrimers could act as collagen mimetics, delivering new proteins to the extra-cellular matrix (e.g., in problems related with tissue engineering).[30]

The synthesis of a new type of biodendrimers and their preliminary screening as agents for drug delivery and nonviral vectors for gene delivery are herein reported.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise noted, all reagents were purchased from commercial sources and used without further purification. Cell lines were obtained from the American Type Culture Collection (Rockville, MD) and media were purchased from ATCC (Manassas, VA) and Invitrogen (Carlsbad, CA). Specific reagents used include: 5-(6)-carboxyfluorescein (CF), from Sigma–Aldrich (Milwaukee, WI), fluorescein isothiocyanate–dextran, 70,000 MW, from Sigma–Aldrich (Milwaukee, WI), supercoiled DNA Ladder 2-10kb and CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay from Promega (Madison, WI), M-PER™ Mammalian Protein Extraction Reagent from Pierce Biotechnology (Rockford, IL), Texas Red–Dextran® (70,000 MW), neutral and ProLong® Antifade Kit (P7481) from Molecular Probes (Eugene, OR), Superfect from Qiagen (Valencia, CA), Beta-Glo™ assay system from Promega (Madsion, WI), and Label IT® Nucleic Acid Labeling Kit from Mirus (Madison, WI); solid supports for peptide synthesis were supplied from Calbiochem–NovaBiochem AG (Laüfelfingen, Switzerland), protected amino acids were from Calbiochem–NovaBiochem AG and Neosystem (Strasbourg, France), and other peptide reagents were from Fluka Chemika (Buchs, Switzerland) and Calbiochem–NovaBiochem AG. Special reagents for peptide synthesis used include: *N*,*N*-bis(*N*′-9-fluoroenylmethoxycarbonyl (Fmoc)-3-aminopropyl)-glycine potassium hemisulfate from Neosystem, 1,3-di-*tert*-butoxycarbonyl (Boc)-2(trifluoromethylsulfonyl)guanidine from Fluka, and Fmoc-PEG_{3400}-OSu from Shearwater Corporation (Hunstville, AL).

Synthesis of Peptide Dendrimers

General Procedures

Fmoc Group Removal. Fmoc group was removed by washing the resin with *N*,*N*-dimethylformamide (DMF) (4 × 1 min), treating the compounds with piperidine-DMF (2:8) (1 × 1 min + 1 × 20 min), and washing the resin again with DMF (4 × 1 min).

4-Methyltrityl (Mtt) Group Removal. Mtt group of Lys was removed by treating the compounds with trifluoroacetic acid (TFA)- triethyl silane (TES)-dichloromethane (DCM) (3:1:96) (2 × 10 min + 1 × 30 min), DCM (5 × 1 min), *N*,*N*′-diisopropylethylamine (DIEA)-DCM (1:19) (2 × 1 min), DMF (5 × 1 min), again with TFA-TES-DCM (3:1:96) (1 × 10 min), again with DIEA-DCM (1:19) (2 × 1 min), and finally by washing the resin with DCM (5 × 1 min) and DMF (5 × 1 min) (This rather tedious protocol assures the removal of the trityl groups that usually remain noncovalently attached to the resin.).

Coupling Efficiencies. Coupling efficiencies were monitored using the ninhydrin[31] test for primary amines or the chloranil[32] and De Clercq[33] tests for secondary amines.

CF Incorporation. CF was coupled (10 equiv) after 1 min of preactivation with *N*,*N*′diisopropylcarbo-diimide (DIPCDI) (10 equiv) in the presence of 1-hydroxybenzotriazole (HOBt) (10 equiv) in DMF for 2 h at 25°C. Once the fluorescent label was incorporated, further manipulations were carried out in the dark. After incorporation of CF, the compounds were treated with piperidine to remove overincorporations of CF.[34]

Acidolytic Cleavage with TFA. Prior to acidolytic cleavage, all peptide resins were washed with DMF (4 × 1 min), DCM (4 × 1 min), MeOH (4 × 1 min), and dried. The cleavage was achieved with TFA-H_2O-TIS (95:2.5 : 2.5) for 2 h at 25°C. In the case of *dendrimers 3, 4*, and *5*, the peptide resins were cleaved with Reagent K [TFA-thioanisole-H_2O-phenol-1,2-ethanedithiol (82.5:5:5:5:2.5)]. Upon resin cleavage all crude peptides were subsequently precipitated with anhydrous methyl *tert*-butyl ether, dissolved in acetic acid, and lyophilized.

RP-HPLC Profiles and (MS). The purity of the crude dendrimers was verified by analytical RP-HPLC using different linear gradients of acetronitrile (MeCN) (containing 0.036% TFA) and H_2O (containing 0.045% TFA) at a flow rate of 1 mL/min. RP-HPLC was performed using an Alliance 2695 Waters Chromatography system with a reverse-phase Symmetry® C18 (3.9 × 15 mm, 5 μm) column with a Waters 996 Photodiode Array Detector.

Appropriate aqueous acetonitrile gradients were found to be: A, 0–100% MeCN; B, 10–70% MeCN; C, 10–60% MeCN; D, 15–45% MeCN; and E, 10–50% MeCN. The same aqueous-acetonitrile gradients were also used for semipreparative RP-HPLC purifications using MeCN (containing 0.1% TFA) and H_2O (containing 0.1% TFA).

Mass spectra of peptides were registered using a matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) and analytical RP-HPLC-MS. Mass spectra were recorded on a MALDI Voyager DE RP TOF (Applied Biosystems, Framingham, MA) using α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, and sinapinic acid as matrices.

RP-HPLC-MS analyses were performed in an HPLC-HS system (HPLC system, Alliance Waters 2795 Chromatography system with Waters 2487 Dual λ Absorbance Detector; masses: mass spectra were recorded on a Micromass ZQ Mass Spectrometer) with appropriate gradients of H_2O (containing 0.1% formic acid) and MeCN (containing 0.07% formic acid).

Purification. Some dendrimers were in large not purified because the purities of crude products were considered acceptable for primary screening of the library. Semipreparative RP-HPLC purifications were performed using a Waters 600 Controller Chromatography system with a reverse-phase Symmetry® C8 (30 × 100 mm, 5 μm) column with appropriate gradients of MeCN (containing 0.1% TFA) and H_2O (containing 0.1% TFA) at a flow rate of 25 mL/min and compounds were detected by a Waters 2487 Dual λ Absorbance Detector at 220 and 443 nm. In the case of *dendrimer 2* and *dendrimer 6*, the purification was carried out at a flow rate of 12 mL/min.

Obtaining the N^{α}(Fmoc)-N^{ε}(Mtt)-lysine-*p*-[(*R,S*)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (AM)-Gly-*p*-methylbenzhydrylamine (MBHA) resin (1). The substitution of the MBHA resin was reduced (from the initial loading 0.7 to 0.18 mmol/g). The incorporation of Fmoc-Glycine-OH (0.5 equiv) was carried out following standard coupling protocols of solid-phase synthesis. After coupling, the resin was washed with DMF (4 × 1 min) and DCM (4 × 1 min) and an aliquot was treated with piperidine-DMF (2:8). Resin loading was monitored by measuring the solution concentration of the liberated dibenzofulvene via uv spectroscopy. Once the desired loading was accomplished, the remaining amino groups on the resin were blocked by acetylation with acetic anhydride (50 equiv) and DIEA (50 equiv) (1 × 30 min). Coupling of Fmoc-AM-OH handle (10 equiv) was then carried out with DIPCDI (10 equiv) in the presence of HOBt (10 equiv) in DMF for 16 h at 25°C. Finally, N^{α}(Fmoc)-N^{ε}(Mtt)-lysine-OH (5 equiv) was introduced with DIPCDI (5 equiv) and HOBt (5 equiv) in DMF for 1 h at 25°C.

Synthesis of Dendrimeric Scaffold ((Fmoc-GlyPro$_5$)$_2$ imidazolidine-2-carboxylic acid (Imd)$_2$-(GlyPro$_5$)$_2$-Imd-X-N^{ε}(Mtt)-lysine-AM-Gly-MBHA Resin (2). X was omitted for *dendrimers 15* and X was incorporated as a monodisperse polyamide pseudo-PEG and polydisperse PEG for *dendrimers 6* and *7*, respectively.

Starting with 270 mg of (**1**), ((Fmoc-GlyPro$_5$)$_2$-Imd)$_2$-(GlyPro$_5$)$_2$-Imd-N^{ε}(Mtt)-lysine-AM-Gly-MBHA resin (**2**) was synthesized on solidphase using an optimized methodology for fragment coupling, purification, and characterization previously described.[29,30] Before modifying the periphery, an aliquot of (**2**) was treated with TFA-H_2O-TIS (95:2.5:2.5) for 1 h at 25°C. Dendrimer purity was characterized by analytical RP-HPLC (using gradient B) and MALDI-TOF. Analytical RP-HPLC-MS using gradient D: calcd $(M+2H)^{2+}$ 1848.68, found 1848.93; $(M+3H)^{3+}$ calcd 1232.8, found 1233.0; $(M+4H)^{4+}$ calcd 924.84, found 925.0; $(M+5H)^{5+}$ calcd 740.1, found 740.24. MALDI: calcd $[M+H]^+$ 3696.36, found $[M+Na]^+$ 3718.39; found $[M+K]^+$ 3734.87. When purity of the dendrimeric scaffold,[1] as determined by analytical RP-HPLC (using gradient B), was approximately 90% the peptide resin was divided into five aliquots, from which the final dendrimers (*dendrimers* 15) were ultimately obtained.

Peptide Dendrimers

***Dendrimer 1*: Free Amine.** A CF was incorporated after removal of the Mtt group of (**2**). The peptide resin was then cleaved providing dendrimer with a purity of 85%, which was verified by analytical RP-HPLC-MS using gradient C: $(M+H)^+$ calcd 4054.67; $(M+3H)^{3+}$ calcd 1352.23, found 1352.44; $(M+4H)^{4+}$ calcd 1014.4, found 1014.85; $(M+5H)^{5+}$ calcd 811.73, found 811.90.

***Dendrimer 2*: Spermidine.** After removal of the Fmoc group of (**2**), *N*,*N*-bis(*N*′-Fmoc-3-aminopropyl)-glycine potassium hemisulfate, liberated from a $KHSO_4$ solution

by organic extraction, was coupled to (24 equiv : 6 equiv per amino group) using DIPCDI (24 equiv) and HOBt (24 equiv) in DMF (2 × 1 h) at 25°C. The Mtt group was then removed and CF was incorporated. The cleavage was performed as described above. The crude dendrimer was purified by semipreparative RP-HPLC using gradient C with a reverse-phase Symmetry® C18 (30 × 100 mm, 5 μm) column to give the title compound with a purity of 80%, as determined by analytical RP-HPLC using gradient C: MALDI calcd $[M+H]^+$ 4739.65, found $[M+Na]^+$ 4763.06.

***Dendrimer 3*: Free Guanidine.** After removal of the Fmoc group of (**2**), 1,3 di-Boc-2(trifluoromethylsulfonyl)-guanidine (different assays were previously performed to verify the orthogonality between the Boc group from the guanidine motif and the Mtt group) was incorporated (24 equiv) using DIEA (12 equiv) in DMF (3 × 2 h + 16 h) at 25°C. The Mtt group was then removed under slightly milder conditions (treatments of 2 min) from those previously used to maintain the integrity of the Boc group. Afterward, CF was introduced as described above. Finally, the peptide dendrimer resin was cleaved with Reagent K and the crude product was purified by semipreparative RP-HPLC using gradient C. The final product was obtained with a purity of 82% as determined by analytical RP-HPLC using gradient B. Dendrimer purity was characterized by analytical RP-HPLC-MS and MALDI-TOF. Analytical RP-HPLC-MS using gradient C: $(M+3H)^{3+}$ calcd 1408.28, found 1408.34; $(M+4H)^{4+}$ calcd 1056.46, found 1056.7; $(M+5H)^{5+}$ calcd 845.37, found 845.46; MALDI calcd $[M+H]^+$ 4222.84, found $[M+Na]^+$ 4247.00, found $[M+K]^+$ 4260.02.

***Dendrimer 4*: Substituted Guanidine.** After removal of the Fmoc group of (**2**), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate (12 equiv) was added using the presence of DIEA (4.4 equiv) in DMF (2 × 2 h + 16 h) at 25°C. CF was then added and cleavage of the peptide dendrimer resin was carried out with Reagent K, affording the crude product with a purity of 55% using gradient B. MALDI calcd $[M+H]^+$ 4655.58, found $[M+K]^+$ 4696.28.

***Dendrimer 5*: Arginine-rich Sequence.** After removal of the Fmoc group of (**2**), a sequence of Arg4 was incorporated stepwise by mediated coupling of Fmoc-Arg(2,2,4,6,7-pentamethylethylchroman-5-sulfonyl)-OH (24 equiv) using DIPCDI (24 equiv) and HOBt (24 equiv) in DMF (2 × 1 h). Mtt removal was achieved under milder conditions as in the case of *dendrimer 3* and CF was subsequently added. Finally, cleavage of the dendrimer from the resin was carried out with Reagent K for 24 h to ensure the complete removal of all Arg protecting groups. The desired dendrimer was obtained with a purity of 75% using gradient B. MALDI calcd $[M+H]^+$ 6553.69, found $[M+Na]^+$ 6571.64. Analytical RP-HPLC-MS by gradient B: $(M+H)^{1+}$ calcd 6553.69; $(M+5H)^{5+}$ calcd 1311.54, found 1311.76; $(M+6H)^{6+}$ calcd 1093.12, found 1093.40; $(M+7H)^{7+}$ calcd 937.10, found 937.11; $(M+8H)^{8+}$ calcd 820.10, found 820.33; $(M+9H)^{9+}$ calcd 729.1, found 729.30; $(M+10H)^{10+}$ calcd 656.27, found 656.52.

***Dendrimer 6*: Free Amine monodisperse PEG.** Starting from (**1**), a monodisperse polyamide pseudo-PEG_{2219} The synthesis of pseudo-PEG_{2219} was carried out without success using the building blocks glycolic anhydride and PEG_{220}. was attached stepwise using the methodology described by Rose and Vizzavona[35] The dendrimer was synthesized using the methodology described in the general procedures for the scaffold (**2**). After Mtt removal and subsequent incorporation of CF, the peptide dendrimer was cleaved and purified by semipreparative RP-HPLC with a reverse-phase Symmetry® C18 (30 × 100 mm, 5 μm) column using gradient E to afford the title compound with a purity of apporximately 90% using gradient B. MALDI calcd $[M+H]^+$ 6269.48, found $[M+H]^+$ 6267.82; $[M+Na]^+$ found 6290.79; found $[M+K]^+$ 6307.83.

***Dendrimer 7*: Free Amine–PEG Polydisperse.** After removal of the Fmoc group of (**1**), the coupling of Fmoc-PEG_{3400}OSu (3 equiv) was accomplished with DIEA (3 equiv) in DMF (1 × 2 h). Dendrimer was then synthesized using the methodology described in general procedures for the scaffold (**2**). The Mtt group was removed, a CF was introduced, and the peptide resin was then cleaved. The crude dendrimer was obtained with adequate polydispersity as observed by MALDI. MALDI calcd $[M+H]^+$ 7429, found $[M+H]^+$ 7428.

***Linear 8*: Pro_{14}.** Starting from (**1**), the linear peptide was synthesized by sequential incorporation of two Fmoc-Pro_7-OH fragments (2 equiv) using benzotriazol oxytris-(pyrrolidino)phosphonium hexafluorophosphate (2 equiv) and DIEA (4 equiv) in DMF for 2 h at 25°C. After Mtt removal and subsequent introduction of a CF, the peptide resin was cleaved. The crude product was purified by semipreparative RP-HPLC using gradient C to provide a 96% pure peptide by gradient B. MALDI calcd $[M+H]^+$ 1864.16, found $[M+Na]^+$ 1886.15; found $[M+K]^+$ 1902.07; Analytical RP-HPLC-MS: $(M+H)^{1+}$ calcd 1864.16, found 1864.41.

***Linear 9*: Pro_{14} polydisperse PEG.** Polydisperse PEG_{3400} was coupled onto (**1**) as performed in *dendrimer 7* and with the same conditions as linear 8 for the incorporation of Pro_{14}. The peptide resin was cleaved and the crude dendrimer obtained was found to possess the adequate polydispersity as observed by MALDI. MALDI calcd $[M+H]^+$ 5239, found $[M+H]^+$ 5235.

Cell Lines and Culture Conditions. The following cell lines were routinely grown at 37°C in a humidified atmosphere with 5% CO_2: HeLa S-3 [epithelial *Homo sapiens* (human)] in MEM (Eagle) supplemented with 10% (v/v) FBS, Jaws II (Dendritic Cells) in Alpha minimum essential medium supplemented with 4 mM l-glutamine, 1 mM sodium pyruvate, and 5 ng/mL of GMCSF and 20% FBS and COS-7 [fibroblast *Cercopithecus aethiops* (African green monkey)] in DMEM supplemented with 10% (v/v) FBS.

Cellular Uptake of Peptide. The cells (5 × 10^4 HeLa cells/well, 2.4 × 10^4 COS-7 cells/well and 8 × 10^4 DC cells/well) were seeded in 96-well microplates and incubated for 24 h (20 h in the case of HeLa) before peptide solutions

were added. After removal of the medium, the cells were exposed to peptide dendrimer solutions (100 μL) prepared in serum-free medium with concentrations varying from 1 to 60 μM at 37°C. At selected time intervals of incubation, cells were washed three times with PBS to remove any excess dendrimer from the extra-cellular space and 100 μL of lysis buffer (MPER® mammalian protein extraction reagent) was added to the cells for 20 min. The supernatant was transferred to black 96-well microplates, and the internalized carboxyfluorescein-labeled peptide dendrimers were fluorometrically quantified using an excitation wavelength of 495 nm and an emission wavelength of 520 nm. Reported values of internalization represent the average of triplicate wells and experiments were performed at least three times.

Confocal Laser Microscopy. HeLa cells (2×10^5 cells/well) were plated onto a glass coverslip, cultured for 20 h, and then incubated with peptide dendrimer solutions in serum-free medium for 2 h at 37°C. In the case of low temperature assays, cells were preincubated for 30 min at 4°C.

For peptide dendrimer cellular distribution experiments, cells were incubated with a solution containing peptide dendrimers and Texas Red–Dextran (5 mg/mL) (1:20, v/v) for 2 h at 37°C.

For peptide dendrimer–DNA complex (20:1) cellular internalization experiments, HeLa cells were exposed to the complex for varying amounts of time at 37°C. DNA was labeled with rhodamine following the Label IT® CX-Rhodamine Labeling Kit protocol.

All of the experiments described above were performed in dark conditions. After removal of the peptide-containing medium, the cells were washed three times with PBS and then fixed with 4% (w/v) paraformaldehyde in PBS at 4°C. Finally, cells were washed three times with PBS and the slides were carefully mounted in a ProLong® Antifade Kit (P-7481). Microscopic examination was performed on a ZEISS confocal microscope (magnification ×63W) using LSM 510 Software.

Cytotoxicity

Cell Viability. The quantitative (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) colorimetric assay was used to determine the cytotoxicity of the peptide dendrimers. The HeLa cells (2×10^4cells/well) were plated on 96-well microplates for 20 h. After removal of the medium the cells were rinsed with warm PBS and incubated with 0.1 mL of peptide dendrimer (60 μM) dissolved in the serum-free medium for 2 h at 37°C. The cells were then washed with PBS and incubated with 0.1 mL of fresh medium containing MTS solution (20 μL/well) for another 2 h. Dose–reponse studies of dendrimers *1, 5*, and *7* were performed as described above with the exception that the cells were exposed to increasing concentrations of peptide dendrimer (0.4 to 100 μM). The optical density of each well was measured at 490 nm using a microplate reader. Absorbance was translated into a number of cells according to a standard calibration curve (absorbance versus number of cells) for each experiment. The percentage of cell viability was defined as % cell viability = [x treated cells/x untreated cells] × 100, where x stands for cell numbers. The values for cell viability reported are the average of triplicate wells in experiments performed three times.

Gel Mobility Shift Assay. Mixtures with DNA to dendrimer ratios ranging from 1 to 200 (by mass) were obtained by combining plasmid DNA (pDNA) (0.05 μg) with increasing amounts of peptide dendrimers (1 mg/mL).

The mixtures were incubated for 20 min at room temperature to allow optimal complex. Samples were resolved by 1% agarose gel in 0.89 *M* Tris, 0.89 *M* boric acid and 0.02 *M* EDTA in aqueous solution buffer. The gel was stained with Orange SYBR for 20 min and then visualized under uv light and photographed.

Gels of *dendrimer 5*pVAX1/*Lac Z* (0.1 μg) complexes with different ratios were prepared as described above, but in the presence of DMEM with 10% FBS.

In Vitro Cell Transfection. pVAX1/Lac *Z* encoding β-galactosidase was used for evaluating the efficiency of gene transfer by dendrimers. HeLa cells were plated at 1×10^5 cells/well on 24-well microplates for 20 h before gene transfer. 1 μg of DNA at 100 μg/mL concentration was added to 19 μL of dendrimer solutions of varying concentrations and the mixtures were vortexed. The resulting solutions were incubated for 20 min and mixed with 171 μL of Opti-MEM. Cells were washed once with cold PBS, and approximately 200 μL DNA–dendrimer complex solution was added to each well. After 2 h of incubation, 800 μL of complete culture medium was added to each well. Cells were then incubated for 48 h and subsequently assayed for gene expression. Reported values of transfection represent the average of triplicate wells with experiments performed at least three times. In the case of experiments in the presence of serum, dendrimer–DNA solutions were incubated in 171 μL of MEM (Eagle) supplemented with 10% (v/v) FBS and treated following the same procedure as described before. These transfection values represent the average of duplicates due to the lack of material.

Gene Expression. Transfected cells were washed once with ice-cold PBS and lysed with 150 μL MPER® mammalian protein extraction reagent for 30 min. The solutions were spun at 10,000 rpm at 4°C for 10 min. β-Galactosidase expression was quantified by the BetaGlo™ Assay System following the product instructions. 100 μL of supernatants were mixed with 100 μL of the BetaGlo™ assaying reagent. Samples were incubated at room temperature for 50 min and analyzed in a luminometer. The quantity of β-galactosidase produced was calculated once specific activity of the enzyme was determined from a calibration curve. β-Galactosidase expression was normalized as pg of β-galactosidase per mg of total protein.

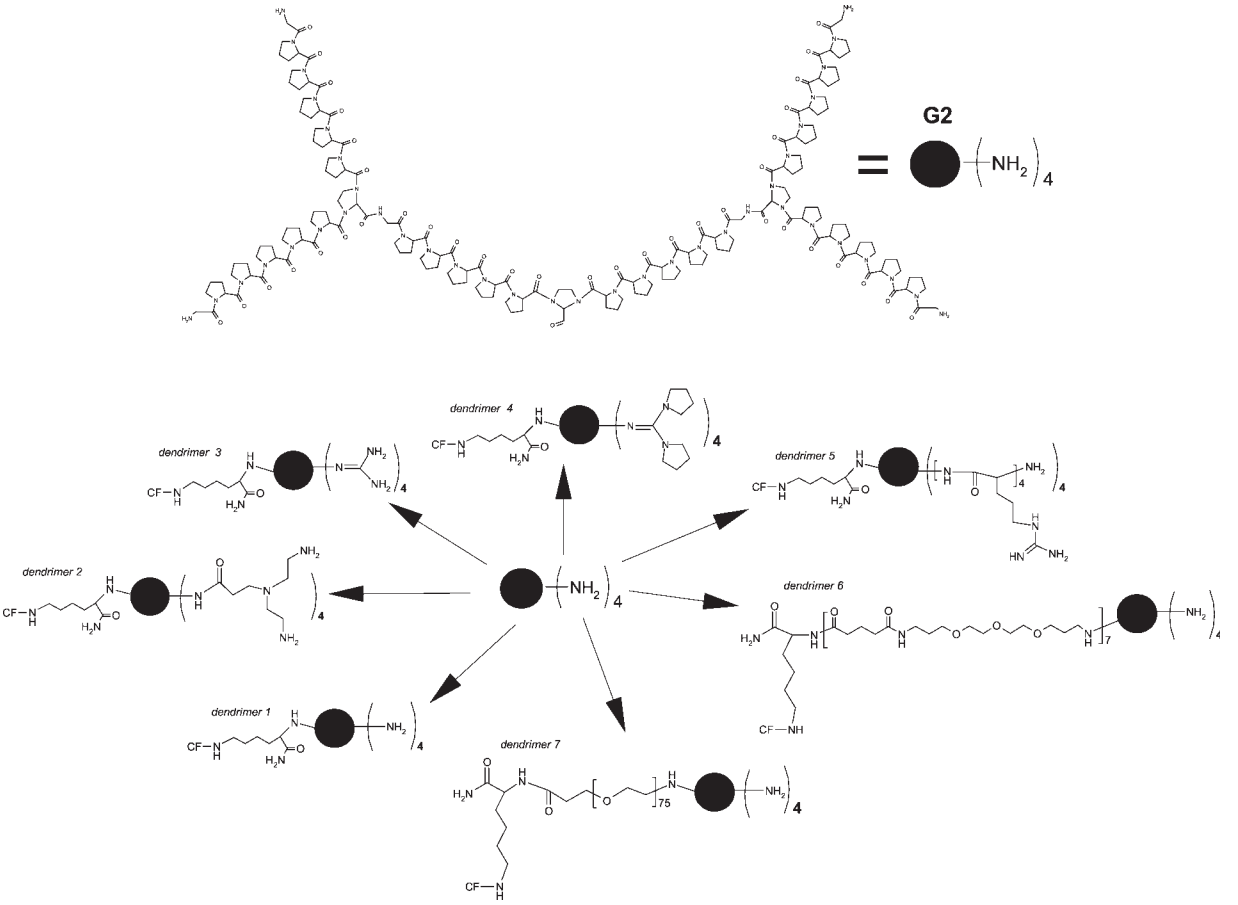

FIGURE 1 Structure of peptide dendrimers.

RESULTS AND DISCUSSION

Synthesis of Peptide Dendrimers

A small library of seven dendrimers (Figure 1) was synthesized via an optimized solid-phase strategy previously developed for the preparation of the first- and second-generation proline dendrimers.[36–38] All dendrimers consisted of a second-generation dendritic template from proline sequences. Dendrimers surfaces were then modified with different positively charged groups. A fluorescent label was covalently attached to the peptides to facilitate detection.

In previous work, first-generation proline dendrimers and linear polyproline peptides were synthesized and their translocation properties were demonstrated.[38] Both linear and dendritic polyprolines were found to be actively internalized by rat kidney cells (NRK-49F). Taking into account these preliminary results of translocation efficiency [Crespo et al.[37] and unpublished data, presented at the 27th European Peptide Symposium, Italy, 2000] for the case of *dendrimers 1*, *2*, and *3*, positively charged groups were introduced to develop carriers able to be internalized and to interact with polyanions such as DNA or oligonucleotides.[39] The Argrich sequence *dendrimer 5* was synthesized to generate vehicles capable of accumulating in the nucleus of mammalian cells.[40] Although fully substituted guanidines are not common in nature, their presence can improve binding to complex receptors;[41] hence, *dendrimer 4* was constructed to study the combination of a proline dendrimer with a hindered guanidine moiety. To avoid hemolytic problems related to carriers during circulation in the blood stream, a PEG chain was added to the C-terminal of the dendrimer.[42] Thus, *dendrimer 7*, with a polydisperse PEG chain, and *dendrimer 6*, with a monodisperse chain, were synthesized to study the effects of the presence and nature of an added PEG group on compound internalization. Finally, with the aim of determining the role played by the dendrimeric structure, syntheses of linear proline peptide without PEG (*linear 8*) and with PEG (*linear 9*) were performed.

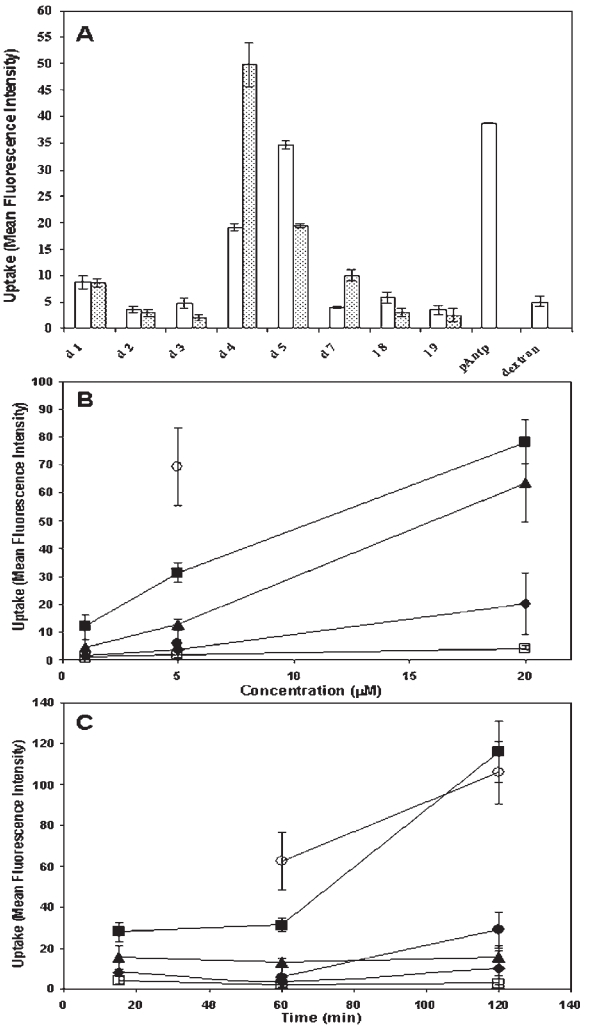

FIGURE 2 (A) Cellular uptake of peptide dendrimers with HeLa cells at 37°C. Fluoresceinated peptides were incubated at 20 and 60 μM for 2 h. (COS-7 and DC cell experiments not shown). Open bars show peptide uptake at 20 μM of peptides with penetratin as a positive control. Shaded bars show peptide uptake at 60 μM. Samples: d1, *dendrimer 1*; d2, *dendrimer 2*; d3, *dendrimer 3*; d4, *dendrimer 4*; d5, dendrimer *5*; d7, *dendrimer 7*; l8, *linear 8*; l9, *linear 9*; pAntp, penetratin; dextran, FITC–dextran. (Each bar represents the mean intensity value ± SE, $n = 3$). At each concentration, a background intensity value was established by measuring the fluorescence of cells incubated with CF. Intensity values for each compound were then calculated by subtracting the appropriate background fluorescence from the observed value for a given compound. (B) Concentration dependence of dendrimer uptake. The *dendrimers 5*, *1*, and *4* were incubated with HeLa cells for 1 h at 37°C at various concentrations. Samples: ○, pAntp; ■, *dendrimer 5*; ▲, *dendrimer 4*; ◆, *dendrimer 1*; ●, FITC–dextran; □, CF. (Each datum represents the mean intensity value ± SE, $n = 3$). A background intensity value was established by measuring the fluorescence of cells incubated with CF (1 μM). Intensity values for each compound were then calculated by subtracting the appropriate background fluorescence from the observed value for a given compound. (C) Time course of dendrimer uptake. *Dendrimers 5*, *1,* and *4* were incubated at 5 μM with HeLa cells for various periods at 37°C. Samples: ○, pAntp; ■, *dendrimer 5*; ▲, *dendrimer 4*; ◆, *dendrimer 1*; ○, FITC–dextran; □, CF. (Each datum expresses average ± SE, $n = 3$).

Cellular Uptake of Peptide Dendrimers

Numerous examples of macromolecules capable of crossing cell membranes and delivering cargo have been reported, such as cell penetrating peptides (CPPs),[43,44] microspheres,[45] loligomers,[46] and dendrimers.[47,48] Myriad factors can influence this complicated translocation process, including cell type, uptake temperature, peptide dendrimer concentration, exposure time, and composition of the medium. All of the aforementioned factors were investigated in this work.

The specific sequence of the *Antennapedia* protein of *Drosophila* (CF-RQIKIWFQNRRMKWKK-$CONH_2$, pAntp, *penetratin*) was selected as a positive control due to the fact that its ability to translocate through cell membranes is well characterized.[49] A fluid phase marker, FITC–Dextran (70 KDa),[50] was also selected as a control.

The penetrating capacity of the library in different cell types was first studied. Three types of cells, HeLa, COS-7, and Jaws II were used. HeLa and COS-7 cells are widely used for testing various delivery vehicles, in particular gene carriers. Jaws II cells are immature mouse dendritic cells. Efficient delivery of antigen to dendritic cells is essential for successful preventive or therapeutic vaccination. We observed that the uptake of this family of polymers was not cell type dependent (data not shown). With this in mind, HeLa cells were chosen to perform the remaining assays.

As illustrated in Figure 2A, *dendrimers 5* and *4* showed the highest translocation, with values comparable to the *penetratin* (pAntp) positive control. *Dendrimer 1*, which could be considered to be the pattern scaffold of the library, and *dendrimer 7* also showed also translocation properties. The modified *dendrimers 2*, *3*, *4,* and *5* differ in their net charge. Surface modifications with guanidine (*dendrimer 3*) and spermidine (*dendrimer 2*) motifs did not increase the uptake, although the compounds exhibit a larger positive net charge in comparison to *dendrimer 1*. In contrast, those dendrimers containing substituted guanidine (*dendrimer 4*) and an Arg-rich sequence (*dendrimer 5*) showed elevated intracellular distribution. Hence, while the presence of a net positive charge

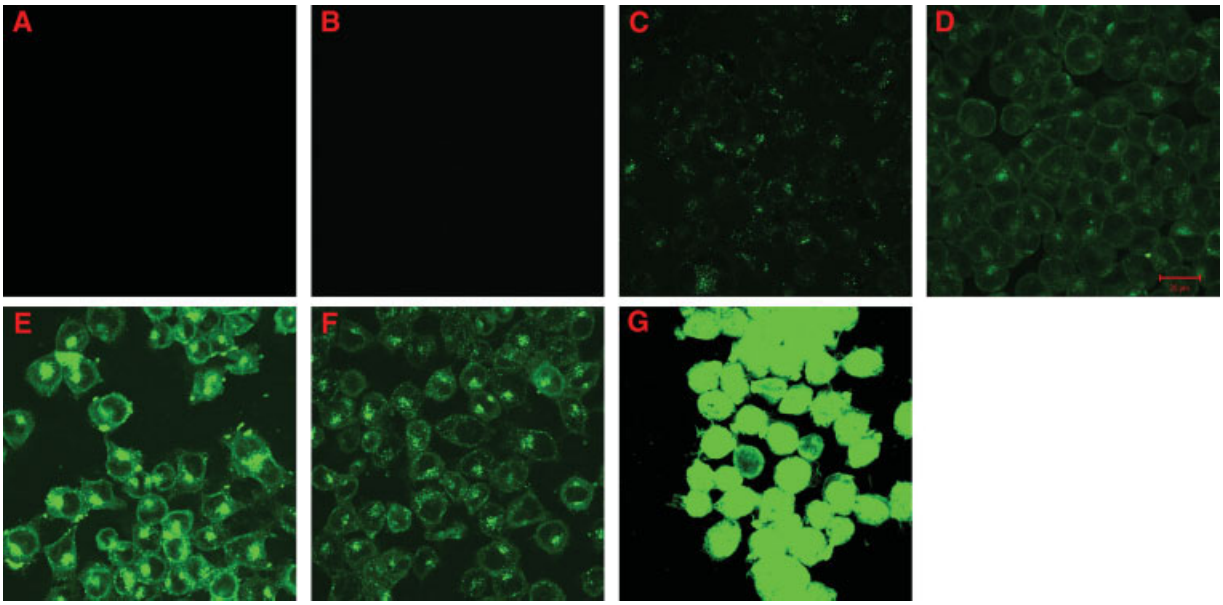

FIGURE 3 Differences in the translocation efficiency and intracellular distribution of dendrimers observed in cells treated with peptide dendrimers, as visualized by confocal microscopy. HeLa cells were incubated for 2 h at 37°C (60 μM each with the exception of *dendrimer 4*, at 20 μM). (A) Control of cells with CF 60 μM. The same profile was observed for untreated cells and cells with CF at 20 μM; (B) *dendrimer 3*. The same profile was observed for *dendrimer 6, linear peptides 8* and *9*; (C) *dendrimer 2*, (D) *dendrimer 7*; (E) *dendrimer 1*; (F) *dendrimer 4*; and (G) *dendrimer 5*.

alone is not sufficient for compound internalization, a high content of basic amino acids does allow penetration. Similar behavior is observed with some CPPs, such as the Arg-rich peptides.[51] When a higher concentration of *dendrimer 5* (60 μM) was used, a decrease in cellular uptake was observed. This result was due to cell death and is explained below in Figure 7.

These aforementioned results suggest that increasing the basic amino acid content of peptide dendrimers does not necessarily cause increased internalization. Although PEG is known to associate with the phospholipid headgroup of the cell membranes, which may facilitate penetration into cells,[52] *dendrimers 7* and *6*, both containing PEG chains, did not show improved uptake compared to *dendrimer 1*. In conclusion, the presence of PEG chains did not improve the translocation of proline dendrimers into cells.

To establish whether internalization of the peptides was concentration dependent, the amount of peptides taken up in HeLa cells was fluorometrically measured (Figure 2B) during 1 h of incubation at 37°C. The assay was performed for the peptide dendrimers with the highest uptake values (*dendrimers 4*, *5*, and *1*). The peptide concentrations used varied from 1 to 20 μM. The uptake of *dendrimer 5* appeared to be related to peptide dendrimer concentration. In contrast, *dendrimers 4* and *1* were much less concentration dependent.

The time course of peptide uptake for *dendrimers 5*, *4*, and *1* was also determined at 5 μM in HeLa cells (Figure 2C). The peptide dendrimers were taken up during the first 2 h, suggesting a saturation effect, with the exception of *dendrimer 5*, which was gradually increased.

Intracellular Distribution

Taking into account the aforementioned results, all dendrimers and linear peptides were incubated with HeLa cells for 2 h at 37°C and the intracellular distributions were examined by confocal laser microscopy (Figure 3). The concentration for all compounds tested was 60 μM (with the exception of *dendrimer 4*, in which 20 μM of solution was tested due to lack of material). Significant differences were observed in the intracellular accumulation and distribution among the compounds tested. Control samples, consisting of cells incubated without peptide or incubated with CF at 60 and 20 μM, did not show any internalization (Figure 3A). Internalization was also not observed for *dendrimers 3* and *6* or for *linear peptides 8* and *9* (Figure 3B). *Dendrimers 2*, *7*, *1*, *4*, and *5* were translo-

cated, with increasing amounts of intracellular compound internalization, respectively (Figure 3C–G). *Dendrimer 5* (the detection parameters for *dendrimer 5* had to be changed due to the enormous signal from the fluorescent marker) exhibited the highest degree of internalization, although *dendrimers 4* and *1* also showed significant internalization. These confocal microscopy results are consistent with the quantitative fluorimetric assay performed above. To confirm intracellular uptake, images were submitted to deconvolution (1020 optical sections spaced by 0.3 μm were taken). Representative photographs for some peptide dendrimers are shown in the supplementary material.

As observed in the images (Figure 3 as well as images provided in the supplementary material), in the case of *dendrimers 4*, *2*, and *7*, the punctuated green dots correspond to vesicular distribution of fluorophore, indicating a possible endocytosis-mediated mechanism. *Dendrimer 1* at 20 μM was incubated using HeLa cells at 37 and 4°C, showing decreased internalization at 4°C (Figure 4).

Dendrimer 1 was also incubated in the presence of Texas Red–Dextran for 2 h. The coincubation with Texas Red–Dextran indicated that *dendrimer 1* had entered the cell by a combination of an endocytosis-mediated mechanism and an unknown mechanism (Figure 5A).

Dendrimer 5 was incubated at a range of concentrations (20, 10, and 5 μM) at 37 and 4°C, resulting in concentration-dependent uptake and reduced internalization at 4°C. *Dendrimer 5* was uniformly distributed inside the cells except for the nucleus, in which it was mostly located in the nucleolus (Figure 6). *Dendrimer 5* was also incubated at a range of concentrations (20, 10, and 5 μM) with Texas Red–Dextran at 37°C, suggesting the coexistence between an endocytosis-mediated mechanism and an unknown mechanism as observed in Figure 5B.

In summary, proline dendrimers (*dendrimers 4*, *2*, and *7*) appear to be internalized by an endocytosis-mediated mechanism. In the case of *dendrimers 5* and *1*, results suggest that different competing mechanisms contribute to the uptake of the peptides. Recent publications provide diverse hypotheses for the translocation of CPPs, suggesting the coexistence of more than one mechanism of uptake and calling for the reevaluation of known mechanisms.[53–56]

Cytotoxicity

Cell viability assays for all peptide dendrimers were performed for 2 h at 60 μM (Figure 7A). Low toxicity was observed for all compounds except *dendrimer 5*. A dose–response study of cytotoxicity was carried out for *dendrimers 5*, *1*, and *7*. The dose responses for both *dendrimers 1* and *7* suggest low toxicity of these materials; cells were incubated with increasing concentrations of peptide dendrimer from 0.4 to 100 μM with no significant cell death observed.

On the other hand, *dendrimer 5* demonstrated a typical toxicity curve; increasing concentration of the Argrich sequence dendrimer resulted in rising cell death with a CD_{50} value of 34 μM. Hence, the potential toxicity of proline dendrimers could be defined by their surface chemistry. For example, *dendrimer 5* is toxic at high concentrations due to the presence of an elevated number of arginine residues. [These results are consistent with other publications that underline the strong toxicity of some CPPs[43] and highly branched polycation polymers such as poly(ethylenimine)[57]].

Dendrimers as Gene Delivery Agents

Experiments were performed to determine whether these dendrimers could deliver genetic material to the insides of cells. Thus, the interaction of peptide dendrimers with a chosen DNA fragment and its gene expression were investigated.

Gel Mobility Shift Assay. Owing to the fact that complexes can form between cationic polymers and the negatively charged phosphate backbone of DNA, the nature of the interaction between pDNA and proline dendrimers was first studied. Hence, a reverse gel was used and the results confirmed that proline peptide dendrimers were cationic polymers with varying net positive charges (data not shown). The peptide dendrimer–DNA complexes (0.05 μg of plasmid pcDNA3/ LDHC4 per well) were prepared under various peptide dendrimer–DNA ratios by mass (Polymer–DNA weight ratios were used instead of charge ratios to specify the composition of complexes because the overall charge on a polyamine varies with pH and temperature.), which ranged from 1 to 200. The resulting complexes were observed on agarose gels. If a dendriplex [this term is usually used to describe the polyplex formed by a polycation (dendrimer) and an anionic oligonucleotide or plasmid] was formed by the addition of the peptide dendrimers, the migration of pDNA would be retarded. Only *dendrimer 5* was able to form a complex with pDNA. The mobility of pDNA was not altered by adding other peptide dendrimers, even when the peptide–DNA ratios were increased to 200 (Figure 8A,B). Therefore, cationic character is not the only factor affecting the formation of polyplexes.

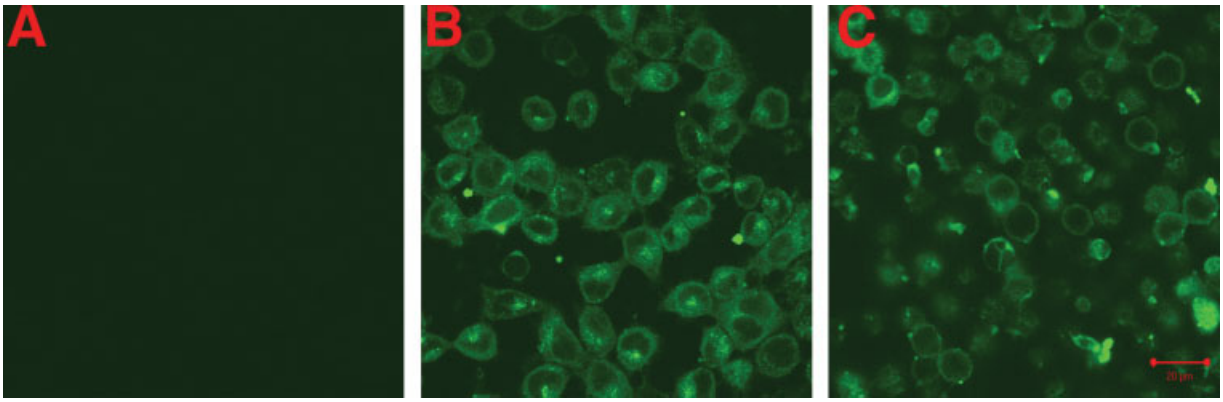

FIGURE 4 Intracellular distribution of *dendrimer 1* observed at different temperatures, as visualized by confocal laser microscopy. HeLa cells were incubated with (A) CF (20 μM) at 37°C, (B) *dendrimer 1* (20 μM) at 37°C, and (C) *dendrimer 1* (20 μM) at 4°C. The same profile was observed for CF (20 μM) at 4°C (not shown) as for CF (20 μM) at 37°C.

A gel retardation assay with increasing ratios of *dendrimer 5*–DNA was carried out to obtain total retardation of the plasmid (Figure 8C). DNA was slightly retarded at a ratio of 1:1 and totally retarded at ratios greater than 2:1. Another gel assay was performed with different ratios of *dendrimer 5*–pVAX1/*Lac Z* mixed in DMEM with 10% FBS to determine whether *dendrimer 5* was able to complex with plas-

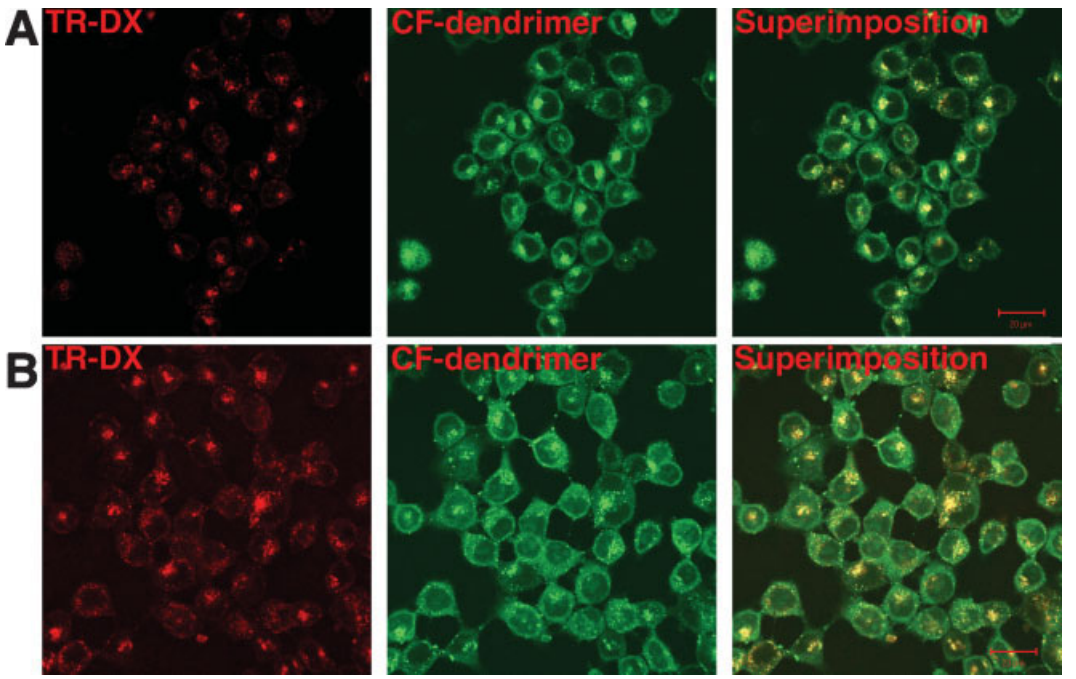

FIGURE 5 Internalization of a solution containing peptide dendrimer coincubated with Texas Red–Dextran in HeLa cells for 2 h at 37°C, as visualized by confocal microscopy. (A) Coincubation of *dendrimer 1* (20 μM) and Texas Red–Dextran, fluorescence of Texas Red–Dextran is indicated by red (left) whereas fluorescence of CF-labeled dendrimer is indicated by green (middle) and their colocalization is seen as yellow (right); (B) coincubation of *dendrimer 5* (5 μM) and Texas Red–Dextran , fluorescence of Texas Red–Dextran is indicated by red (left) whereas fluorescence of CF-labeled dendrimer is indicated by green (center) and their colocalization is seen as yellow (right). The same profile was observed when the coincubation was carried out with Texas Red–Dextran and *dendrimer 5* at both 20 and 10 μM.

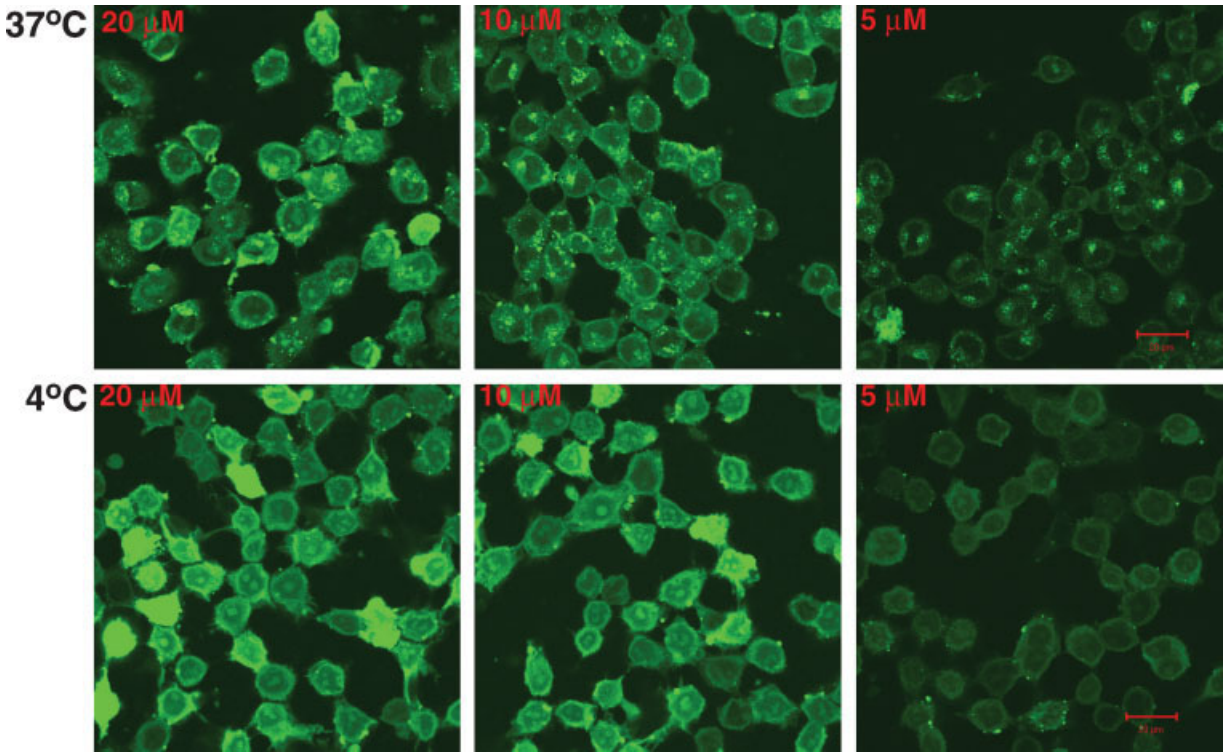

FIGURE 6 Effects of temperature and concentration on cellular uptake of *dendrimer 5* as visualized by confocal laser microscopy. HeLa cells were incubated with dendrimer (20, 10, and 5 μM) at 37 and 4°C, respectively.

mids in the presence of complete cell culture medium. The dendriplex obtained was stable in the presence of medium and indifferent to the order of addition [which can be critical for other combinations of plasmids and nonviral vectors[16]] (Figure 8D).

Taking the aforementioned data into account, it can be postulated that good nonviral vectors capable of forming polyplexes must be (i) cationic polymers with protonable amines at physiological pH and (ii) hydrophilic polymers to decrease nonspecific interactions with biological components.[58] While *dendrimers 2*, *3*, *4*, and *5* show both characteristics, *dendrimers 1*, *6*, and *7* present low cationic character and *linear 8* and *9* do not have cationic conduct. Polyplexes are only formed by *dendrimer 5* at weight ratios of (2:1) and greater.

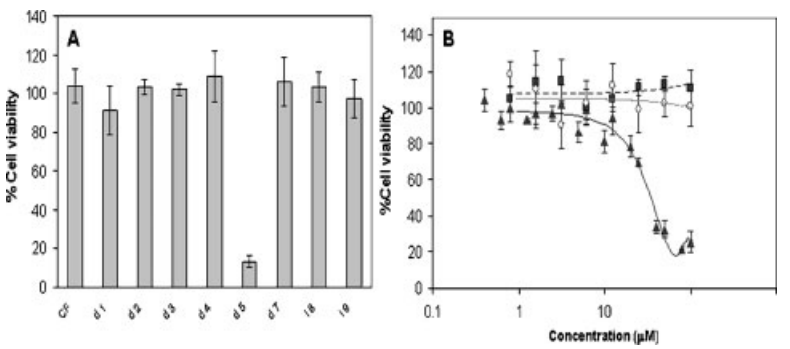

FIGURE 7 (A) Cytotoxicity of peptide dendrimers. HeLa cells were incubated with all peptide dendrimers at 60 μM for 2 h at 37°C. (average ± SE, $n = 3$). (B) Dose response of *dendrimer 1*, *5*, and *7*: HeLa cells were incubated with various concentrations of peptide dendrimers for 2 h at 37°C (average ± SE, $n = 3$). The peptides are represented by triangles (*dendrimer 5*), circles (*dendrimer 7*), and squares (*dendrimer 1*).

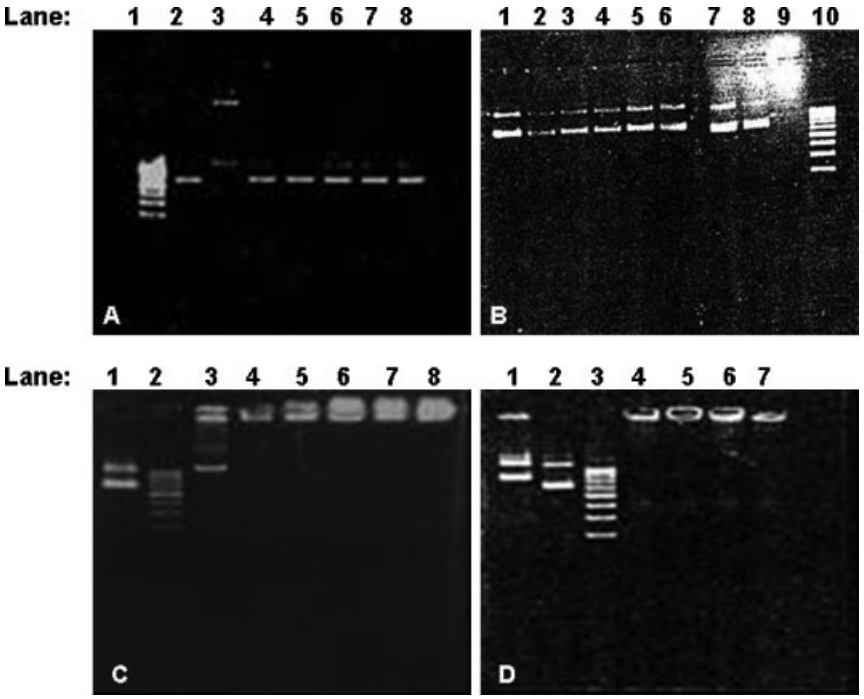

FIGURE 8 Gel retardation assay. (A) Agarose gel of pDNA with peptide dendrimers with weight ratio (1:1). Lane 1, scDNA; lane 2, plasmid pcDNA3/LDHC4; lane 3, *dendrimer 5*:pDNA; lane 4, *dendrimer 3*:pDNA; lane 5, *dendrimer 4*:pDNA; lane 6, dendrimer *2*:pDNA; lane 7, linear *8*:pDNA; lane 8, dendrimer *1*:pDNA. (B) Agarose gel of plasmid pcDNA3/LDH-C4 mixed with increasing amounts of *dendrimer 2* until ratio (100:1): lane 1, Plasmid pcDNA3/LDH-C4; dendrimer–pDNA weight ratios: lane 2 (5:1), lane 3 (10:1), lane 4 (15:1), lane 5 (20:1), lane 6 (30:1), lane 7 (50:1), lane 8 (100:1), lane 9 (excess of *dendrimer 2* alone), lane 10 scDNA. The brightness in lane 9 is due to an excess of fluoresceinated (CF) dendrimer. (Gels of the other dendrimers with increasing ratios and *dendrimer 2* at ratio 200:1 are not shown because they did not provide substantial information). (C) Agarose gel of plasmid pcDNA3/LDH-C4 mixed with increasing amounts of *dendrimer 5*: lane 1, plasmid pcDNA3/LDH-C4; lane 2, scDNA. Dendrimer–pDNA weight ratios: lane 3 (1:1), lane 4 (2:1), lane 5 (4:1), lane 6 (6:1), lane 7 (8:1), lane 8 (10:1). (D) Agarose gel of *dendrimer 5* different ratios with pVAX1/*Lac Z* in DMEM with 10% FBS for in vitro conditions. Lane 1, pVAX1/*Lac Z*; lane 2, plasmid pcDNA3/LDHC4; lane 3, scDNA. Dendrimer-pVAX1/*Lac Z* adding first of all medium: lane 4 (4:1); lane 5 (2:1), and adding medium (DMEM with 10% FBS) at the end: lane 6 (4:1) and lane 7. (2:1).

Cellular Internalization of Peptide Dendrimers–DNA Complexes. The intracellular movement and migration inside nuclear compartments of DNA labeled with rhodamine, complexed with *dendrimer 5*, and incubated with HeLa cells was tracked over time by confocal microscopy (images were taken at 30 min, 2 h, and 24 h). As in Figure 9, the yellow color observed inside of cells highlights areas of comigration between fluorochromes and suggests that the labeled plasmid is coated by dendrimer.

Cells were strongly transfected by dendriplexes and sections of images illustrating their internalization and colocalization were obtained. Cells treated with DNA alone were used as a control and exhibited no signs of internalization. The internalization of dendriplexes is obtained in 30 min and increased at 2 h. After 24 h, few polyplexes are found, thus indicating the strong interaction between *dendrimer 5* and pDNA. It was hence affirmed that dendriplexes are achieved and that when endocytosis is the main route of cellular entry the effective formation of nanometerscale polymer–DNA complexes is a requirement for achieving high levels of cellular uptake.

In Vitro Gene Transfer. Despite the fact that only dendrimer *5* interacted with DNA (see Gel Mobility Shift Assay), transfection experiments were performed with all synthesized polymers at a polymer–DNA ratio of 50:1 (by mass) using a HeLa cell line. None of the polymers mediated appreciable levels of transfection with the exception of *dendrimer 5*. Therefore, *dendrimer 5* became our focus, and the

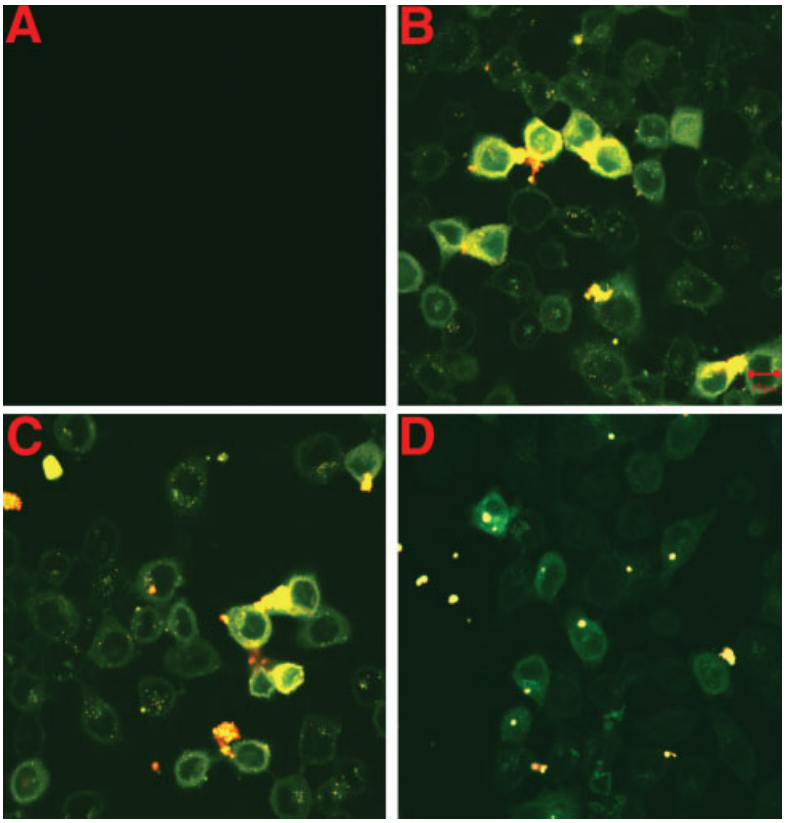

FIGURE 9 Localization of *dendrimer 5*–plasmid complexes examined by confocal laser microscopy in HeLa cells at 37°C. The comigration of labeled *dendrimer 5* (green) and DNA-labeled plasmid (red) generates areas of yellow fluorescence. (A) Cells incubated with Naked DNA rhodamine labeled for 30 min and 2 h (same profile), (B) Intracellular distribution of dendriplexes after 30- min exposure, (C) after 2 h, and (D) after 24 h of exposure.

effect of the ratio of *dendrimer 5* to DNA on the efficiency of gene transfer was then studied, as was the possibility that an appreciable level of gene transfer could be achieved with low cytotoxicity. SuperFect, a commercially available dendrimer-based transfection reagent, was used as a positive control.

As illustrated in Figure 10, gene transfer efficiency peaked at a *dendrimer 5* to DNA ratio (the ratio of arginine groups to phosphate groups on DNA by micromoles) of 25.6 then sharply declined at higher ratios. The *dendrimer 5* to DNA ratio of 25.6 corresponds to 24 μM of *dendrimer 5*, which is two- to eightfold higher than when using SuperFect. Since the CD_{50} of *dendrimer 5* is 34 μM, appreciable gene transfer can be achieved with low cytotoxicity.

To make a preliminary study of the effect of the serum, transfection experiments were repeated in the presence of 10% FBS. The transfection activity was still present, although a decrease of intensity was observed. A similar decrease in transfection efficiency in the presence of serum has been reported for other transfection reagents, such as lipofectamine, a quaternary lipidic ammonium salt.[59] In this particular case, in HeLa cells the transfection efficiency is greater in the absence of serum. On the contrary, for other transfection reagents, such as Superfect, an activated poly(amidoamine) dendrimer, the transfection efficiency remains or even increases in the presence of serum.[60] These dendrimers posses many tertiary groups distributed inside the whole structure,[47,61,62] which become protonated under weakly acid conditions creating a sponge effect.[63] From a structural point of view, *dendrimer 5* resembles SuperFect due to its dendritic structure, but its positively charged guanidinium groups are located at the surface, leading to a molecule with a clear amphipatic properties. As indicated before, the serum effect on the transfection efficiency of *dendrimer 5* is similar to those observed on Lipofectamine, probably due to its amphipatic properties. Careful evaluation of the different factors that affect the transfection could provide an improved proline dendrimer.[64]

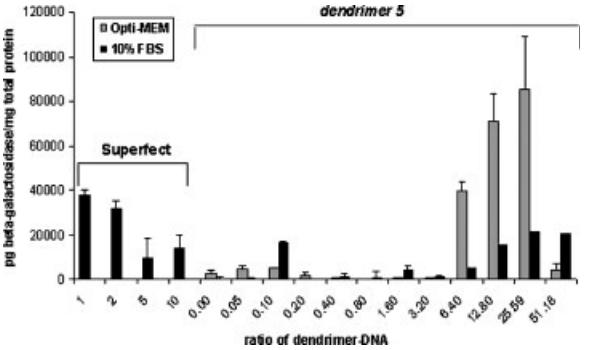

FIGURE 10 Levels of β-galactosidase activity detected in extracts of HeLa cells transfected with dendrimer 5–pVAX1/*Lac Z* complexes and SuperFect as a control.

CONCLUSION

A new class of peptidebased, dendrimeric biopolymers has been synthesized. The ability of these dendrimers to cross the cell membranes of mammalian cells, to self-localize (in the nucleus or cytosol), to form compact dendriplexes, and their toxicity were studied and a compound (*dendrimer* 5) with therapeutic potential was identified. The effects of carrier composition (degree of Pro richness), structure type (linear or dendrimeric), and surface and chain end-group modification on the aforementioned properties were also examined. The results suggest that differences in dendrimer structure translate into distinct internalization and transfection characteristics.

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