Communications

Dendrimer-Based Targeted Delivery of an Apoptotic Sensor in Cancer Cells

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Our previous studies have demonstrated the applicability of poly(amidoamine) (PAMAM) dendrimers as a platform for the targeted delivery of chemotherapeutic drugs both in vitro and in vivo. To monitor the rate and extent of cell-killing caused by the delivered chemotherapeutic drug, we wished to analyze the degree of apoptosis in targeted cells on a real-time basis. As the apoptosis-regulating caspases are activated during the apoptotic process, several caspase-hydrolyzable, fluorescence resonance energy transfer (FRET)-based substrates have been marketed for the detection of apoptosis. However, the applicability of these agents is limited because of their nonspecificity and the consequent high background fluorescence in tissues. Here we show the synthesis, characterization, and in vitro targeting of an engineered PAMAM nanodevice in which folic acid (FA) is conjugated as the targeting molecule and a caspase-specific FRET-based agent (PhiPhiLux G_1D_2) is conjugated as the apoptosis-detecting agent. This conjugate specifically targets FA-receptor-positive, KB cells. In these cells, the apoptosis-inducing agent staurosporine caused a 5-fold increase in the cellular fluorescence. These results show, for the first time, the potential applicability of a targeted apoptosis-measuring nanodevice, which could be used for simultaneously monitoring the apoptotic potential of a delivered drug.

Introduction

Poly(amidoamine) (PAMAM) dendrimers are spherical, highly ordered, multibranched, nanometer-sized macromolecules having positively charged amino groups on the surface at physiological conditions.[1,2] Because of the abundant surface amino groups, a variety of reactions can be performed under mild conditions to generate PAMAM conjugates. Our recent studies have demonstrated the in vitro and in vivo of generation 5 (G5) PAMAM as a targeting agent using targeting molecules such as folic acid (FA),[3−6] antibodies,[7,8] and RGD peptides.[9] In addition, the chemotherapeutic drug methotrexate conjugated to the G5 PAMAM induces specific tumor-cell cytotoxicity both in vitro[4] and in vivo.[6]

The extent of cell death induced by a chemotherapeutic drug is routinely monitored by quantification of in vitro cell viability and growth using XTT and protein assays or specific uptake of dyes such as propidium iodide by dead cells and by measuring the degree of apoptosis using a variety of techniques based on specific apoptosis-induced cellular changes. These changes include nuclear DNA fragmentation, mitochondrial membrane potential, flipping of membrane phospatidyl serine (PS), and activation of cytosolic cysteine proteases termed as caspases.[10] In vivo apoptosis can be quantified by methods such as the binding of radioactive Annexin V, which binds to flipped PS,[11] or the use of a fluorescence resonance energy transfer (FRET)-based dye that becomes fluorescent in cells due to the actions of activated caspases.[12]

Caspase-3 is one of the cysteine proteases most frequently activated during the process of apoptosis. The pro-Caspase-3 is a 32 kDa protein and is processed to the active enzyme that consists of two subunits of 17 and 12 kDa, which in turn cleave proteins that contain the sequence valine−aspartic acid[13] and are essential for the progression of apoptosis. Several FRET-based fluorogenic substrates have been developed to detect active caspase-3 in cells.[14,15] In the absence of active capase-3, these substrates remain nonfluorescent due to energy transfer between donor and acceptor subunits linked through an oligopeptide. In apoptotic cells, active caspase-3 cleaves the oligopeptide between valine and aspartic acid, releasing the fluorescent module. The amount of fluorescence can then be quantified by various detecting techniques.[14−16]

Although current FRET-based methods have the advantage of specifically detecting cells that are apoptotic, these methods do not discern between normal cells and tumor cells. In addition, as the free apoptotic sensor is nonspecifically distributed in all cells and tissues, the effective concentration in the cells of interest may vary during the period of measurement. Therefore it is important that a method is developed to specifically deliver an apoptosis-sensing device simultaneously into the desired cells along with a cytotoxic drug to monitor the real-time apoptotic effects of the drug. Here we report the synthesis and the in vitro apoptosis-sensing function of a bifunctional G5-PAMAM-based

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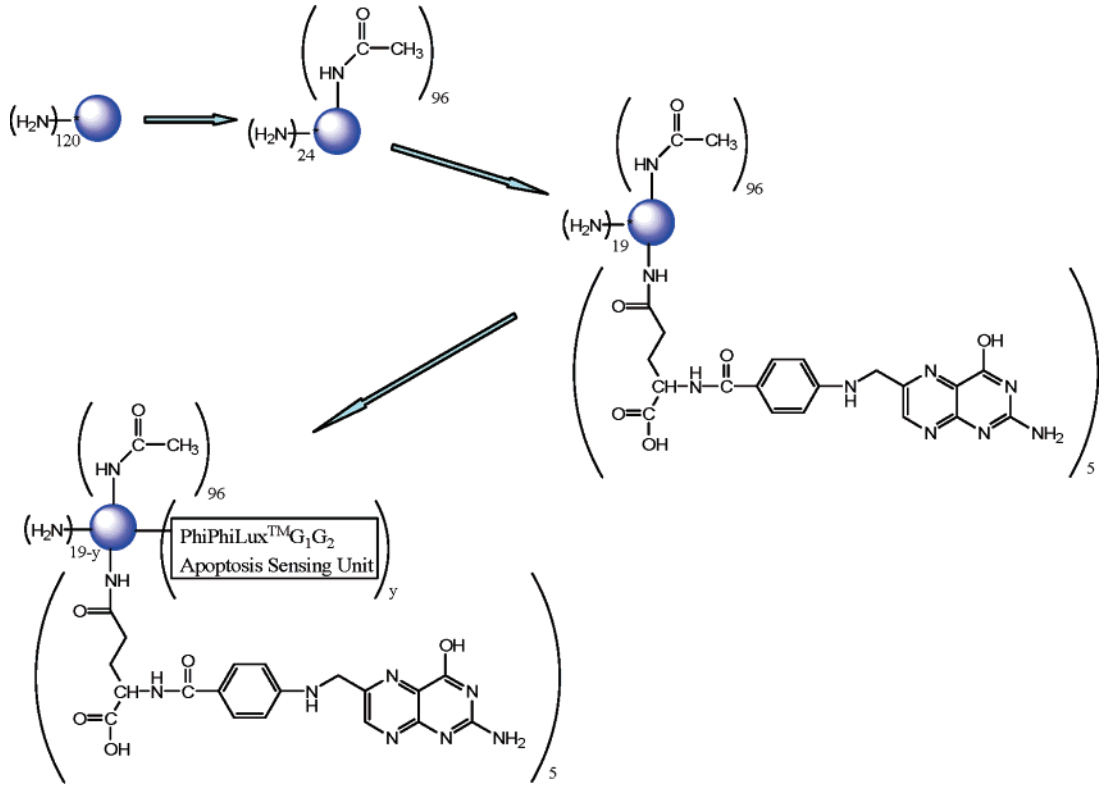
Scheme 1. Synthetic Scheme for Bifunctional PAMAM Dendritic Devices[a]



[a] Order of syntheses: (1) G5 carrier; (2) G5-Ac(96); (3) G5-Ac(96)-FA; (4) G5-Ac(96)-FA-PhiPhiLux G_1D_2

dendrimer nanodevice in which FA was used as the targeting agent and the FRET reagent "PhiPhiLux G_1D_2" was used as the apoptosis-detecting reagent.

Experimental Procedures

Materials. Methanol (MeOH, HPLC grade), acetic anhydride (99%), triethylamine (99.5%), dimethylsulfoxide (DMSO, 99.9%), dimethylformamide (DMF, 99.8%), 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide HCl (EDC, 98%), citric acid (99.5%), sodium azide (99.99%), D_2O, NaCl, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Aldrich Co. The FA and staurosporine were from Sigma (St. Louis, MO). Spectra/Por, dialysis membrane (molecular weight cutoff 3500), Millipor Centricon ultrafiltration membrane YM-10, and phosphate-buffered saline (PBS, pH 7.4) were purchased from Fisher. PhiPhiLux G_1D_2 was from Calbiochem (San Diego, CA). The Jurkat E6 and KB cell lines were from type American Type Culture Collection (ATCC, Manassas, VA), and the UMSCC-38 head and neck squamous carcinoma cell line was kindly provided by Dr. J. Mulè (University of Michigan).

Syntheses of G5-Ac-FA-PhiPhiLux G_1D_2. The synthetic scheme for the production of dendritic devices is given in Scheme 1.

The G5 PAMAM dendrimer (Scheme 1) was synthesized and characterized at the Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS), University of Michigan. The synthesized dendrimer was analyzed by using NMR, high-performance liquid chromatography (HPLC), gel permeation chromatograph (GPC), and potentiometric titration.[5] The molecular weight was found to be 27 500 g/mol by GPC, and the average number of primary amino groups was determined by potentiometric titration to be 120.

Acetylation of G5 PAMAM. Approximately 0.2071 g (7.85×10^{-6} mol) of G5 PAMAM dendrimer in 16 mL of absolute methanol (MeOH) was allowed to react with 59.3 μL (6.28×10^{-4} mol) of acetic anhydride in the presence of 109.4 μL (7.85×10^{-4} mol, 25% molar excess) of triethylamine for 14 h. After intensive dialysis in deionized (DI) water and lyophilization, the yield of the product (Scheme 1, G5-Ac(96)) was 223.0 mg (93.4%). The average number of acetyl groups (96) was determined based on ^{1}H NMR calibration.[5]

Synthesis of G5-Ac(96)-FA. FA was attached to G5-Ac(96) in two successive reactions. A total of 0.0028 g (6.343×10^{-6} mol) of FA was allowed to react with a 14-fold excess of EDC (0.01707 g, 8.906×10^{-5} mol) in a solvent mixture of 3 mL of DMF and 1 mL of DMSO at room temperature for 1 h, and the FA-active ester formed was added dropwise to an aqueous solution of the partially acetylated product G5-Ac(96) (0.0126 g, 4.143×10^{-7} mol) in 12 mL of water, and the reaction time was 3 days at room temperature. After dialysis in DI water, repeated membrane filtration (using PBS and DI water), and lyophilization, the product weight was 0.01209 g (95.45%). The number of FA molecules per dendrimer (5 molecules) was determined by proton NMR spectroscopy. No free FA was observed by using HPLC.

Synthesis of G5-Ac(96)-FA-PhiPhiLux G_1D_2. PhiPhiLux G_1D_2 was attached to the G5-Ac(96)-FA monofunctional dendrimer conjugate in two successive reactions. A total of 0.0013 g (6.685×10^{-7} mol) of PhiPhiLux G_1D_2 (M_w = 1944.73 g/mol) was allowed to react with a 14-fold excess of EDC (0.0018 g, 9.389×10^{-6} mol) in a solvent mixture of 3 mL of DMF and 1 mL of DMSO at room temperature for 1 h. This PhiPhiLux-G_1D_2-active ester solution was added dropwise to an aqueous solution of the partially acetylated monofunctional dendrimer conjugate G5-Ac(96)-FA (0.0023 g, 7.05×10^{-8} mol) in 12 mL of water, and the reaction time was 2 days. After repeated membrane filtration (using PBS and DI water) and lyophilization, the final yield of the product obtained was 0.0033 g. This bifunctional dendrimer conjugate was used to test biological activity and specificity.

Potentiometric Titration. Titration was carried out manually using a Mettler Toledo MP230 pH meter and MicroComb pH electrode at room temperature, 23 ± 1 °C. A 10 mL solution of 0.1 M NaCl was added to precisely weighed 118.4 mg of G5 PAMAM dendrimer to shield amine group interactions. Titration was performed with 0.1037 N HCl, and 0.1033 N NaOH was used for back-titration. The numbers of primary and tertiary amines were determined from back-titration data.

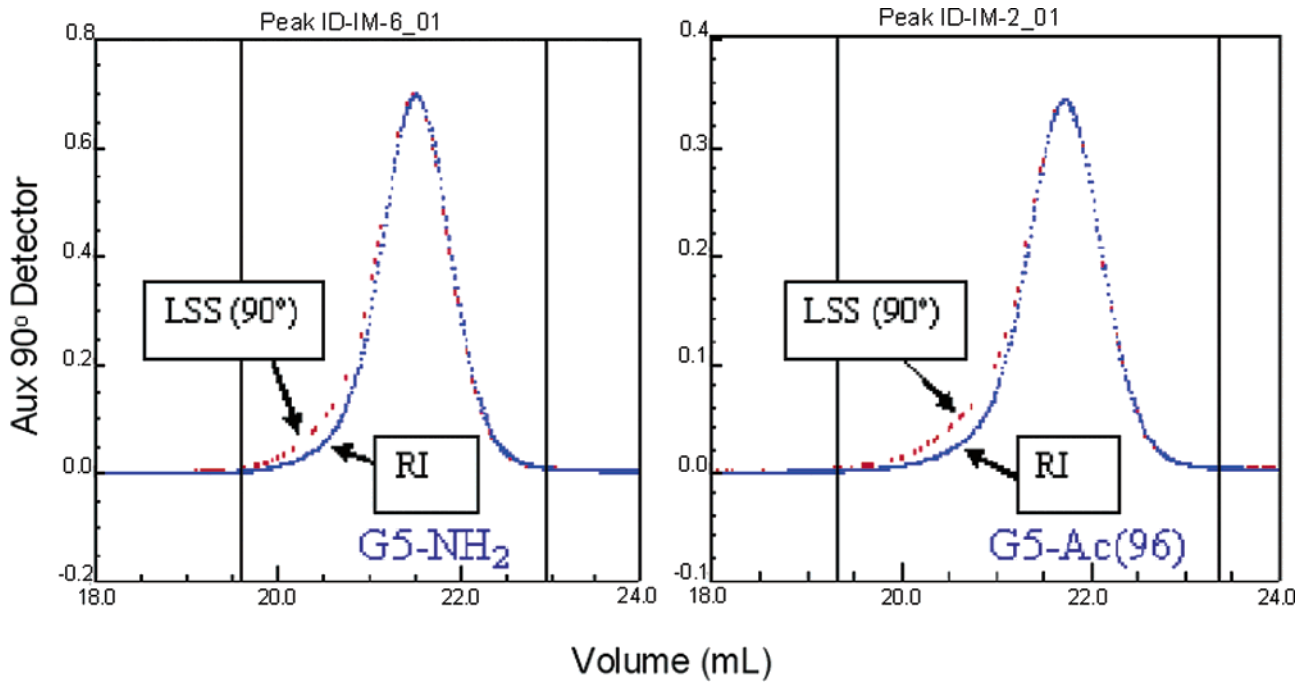


Figure 1. GPC RI and light-scattering signal (90°) of the G5 dendrimer and G5-Ac(96) partially acetylated dendrimer.

Gel Permeation Chromatography. GPC experiments were performed on an Alliance Waters 2690 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt Dawn DSP laser photometer, an Optilab DSP interferometric refractometer (Wyatt Technology Corporation), and TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 μm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 μm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 μm), and G 4000 PW (300 mm × 7.5 mm, 17 μm) columns. Column temperature was maintained at 25 ± 0.1 °C by a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025 wt % sodium azide, pH 2.74, at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL with an injection volume of 100 μL. The molecular weight and molecular weight distribution of the PAMAM dendrimer and its conjugates were determined using Astra 4.7 software (Wyatt Technology Corporation).

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were taken in D_2O and were used to provide integration values for structural analysis by means of a Bruker Avance DRX 500 instrument.

UV Spectrophotometry. UV spectra were recorded in PBS using a Perkin-Elmer UV/vis spectrometer Lambda 20 and Lambda 20 software.

Reverse-Phase HPLC. A reverse-phase ion-pairing HPLC system consisted of a System GOLD 126 solvent module, a model 507 autosampler equipped with a 100 μL loop, and a model 166 UV detector (Beckman Coulter, Fullerton, CA). A Phenomenex (Torrance, CA) Jupiter C5 silica-based HPLC column (250 mm × 4.6 mm, 300 Å) was used for the separation of analytes. Two Phenomenex safety guards were also installed upstream of the HPLC column. The mobile phase for elution of PAMAM dendrimers was a linear gradient beginning with 90:10 water/ acetonitrile (ACN) at a flow rate of 1 mL/min, reaching 50:50 after 30 min. Trifluoroacetic acid (TFA) at a 0.14 wt % concentration in water as well as in ACN was used as a counterion to make the dendrimer−conjugate surfaces hydrophobic. The conjugates were dissolved in the mobile phase (90:10 water/ACN). The injection volume in each case was 50 μL with a sample concentration of approximately 1 mg/mL, and the detection of eluted samples was performed at 210, 242, or 280 nm. The analysis was performed using Beckman's System GOLD Nouveau software.

Characterization of all intermediates has been performed through the use of UV, HPLC, NMR, and GPC, as given above.

Cell Culture and Treatment. The KB cell line is a human epidermoid carcinoma that overexpresses folate receptors, especially when grown in a low-folic-acid medium.[17] The KB and UMSCC (folate-receptor-negative) cells were grown in folic-acid-deficient RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), yielding a final folate concentration roughly that of normal human serum. The Jurkat cells were grown on regular RPMI medium in the presence of 10% heat-inactivated FBS. All of the cells were supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 50 mM L-glutamine and maintained at 37 °C and 5% CO_2.

Approximately 2 × 10^4 Jurkat cells per well were seeded the day before the experiments in 12-well plates with complete medium. An hour before each experiment, the cells were rinsed, 500 μL of fresh medium was added, and the cells were treated with either staurosporine at a concentration of 0.5 μM or PBS (control) for 3.5 h. After treatment, apoptosis was detected using a PhiPhiLux G_1D_2 kit following the vendor's instructions. KB and UMSSC-38 cells were first incubated with either 0.45 μM G5-Ac-FA-PhiPhiLux G_1D_2 or PBS (untreated cells) for 30 min. Then either staurosporine at a concentration of 0.5 μM or PBS (control) was added, and the cells were incubated for an additional 3 h. After incubation the cells were examined under the microscope to observe signs of apoptosis (cell shape changes, membrane blebbing, cytoplasmic shrinkage). Then cells were gently detached from the plate and washed once in PBS supplemented with 0.1% bovine serum albumin (BSA), fluorescence was quantified using a Beckman-Coulter EPICS-XL MCL flow cytometer, and the data were analyzed using Expo32 software (Beckman-Coulter, Miami, FL).

Results and Discussion

The PAMAM dendrimer used in this study was uniform and monodispersed. Determination of molecular weight and the number of primary amino groups was fundamental in designing reactions resulting in the synthesis of a precise conjugate structure.

Potentiometric titration was initially performed to determine the average number of tertiary and primary amino groups. Although the theoretical numbers of primary and tertiary amino groups in the G5-PAMAM are 128,[18,19] the potentiometric analysis gave the values of only 120 primary amines.

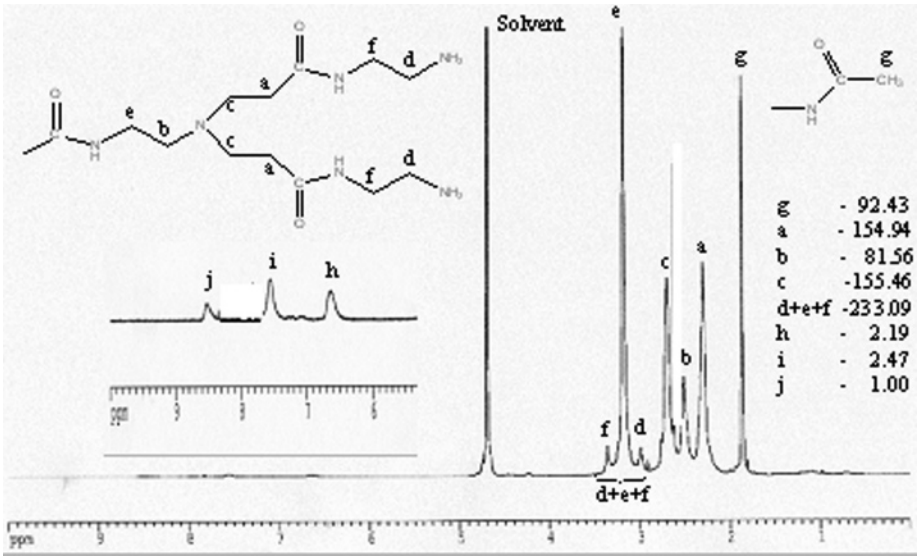


Figure 2. ^{1}H NMR of the G5-Ac(96)-FA(5) conjugate. The inserts depict aromatic peaks that belong to the conjugated FA and integral values of the individual peaks of the dendrimer protons.

Partial acetylation was used to neutralize a fraction of the surface amino groups of the dendrimer device surface to prevent charge-based nonspecific interaction with the negatively charged cell membrane. Leaving a fraction of the primary amines nonacetylated allows for the subsequent attachment of targeting and FRET molecules. Figure 1 shows GPC eluograms of the G5 PAMAM before and after partial acetylation, showing the RI signal and laser light-scattering signal LSS overlapping at 90°, meaning that there is no defect in analyzed structure.

In the next reaction FA was attached to the G5-Ac(96) carrier primarily through the γ-carboxylic group of FA because of its higher reactivity during carbodiimide-mediated coupling to primary amino groups as compared to that of the α-carboxyl group.[3] The affinity of FA for binding to FA receptor (FAR) is not affected when linked through the γ-carboxylic group. NMR was also used to confirm the number of FA molecules attached to the dendrimer (Figure 2). In the case where free FA is present in the sample, sharp peaks would appear in the spectrum (at the broad aromatic peaks). The broadening of the aromatic proton peaks in the G5-Ac(96)-FA spectrum indicates the presence of a covalent bond between FA and the dendrimer. On the basis of the integration values of the methyl protons in the acetamide groups (1.84 ppm) and the aromatic protons in FA (6.64, 7.55, and 8.52 ppm), the number of attached FA molecules was calculated to be 4.9. In a separate study using UV spectroscopy and using a concentration calibration curve of free FA, the number of FA molecules was estimated to be 5.3 (data not shown). Analytical HPLC showed that the G5-Ac(96)-FA(5) conjugate clearly indicates the removal of free FA (1) before and (2) after membrane filtration purification (Figure 3).

To ascertain how the unconjugated PhiPhiLux G_1D_2 functions as an apoptosis-sensing agent, we tested the compound for its ability to quantify staurosporine-induced apoptosis in the Jurkat cells. As shown in Figure 4, control Jurkat cells nonspecifically stained with PhiPhiLux G_1D_2 yielded approximately 34% positive cells (Figure 4B) as compared to unstained control Jurkat cells (Figure 4A). Apoptotic Jurkat cells showed a further increase in fluorescence intensity, yielding approximately 93% positive cells (Figure 4C).

Then PhiPhiLux G_1D_2 (Figure 5) was attached to the G5-Ac(96)-FA dendrimer conjugate as described in the Experimental Procedures section. The purified G5-Ac(96)-FA-PhiPhiLux G_1D_2 nanodevice was examined for its functionality in FAR-positive KB cells and FAR-negative UMSCC-38 cells (as described in the Experimental Procedures section). As shown in Figure 6, control KB cells showed some nonspecific increase in fluorescence intensity as compared to control unstained cells. However, the apoptotic KB cells increased the fluorescence intensity to a much greater degree and were easily distinguished from nonspecifically stained control cells (Figure 6A). In contrast, apoptotic UMSCC-38 cells did not show any increase in fluorescence intensity over the background fluorescence of stained control cells (Figure 6B), suggesting that the nanodevice was not internalized. These results suggest that KB cells actively internalized the nanodevice through folate receptors during the first 30 min of incubation, and after induction of apoptosis, the active caspase-3 cleaved the bond between the donor and the

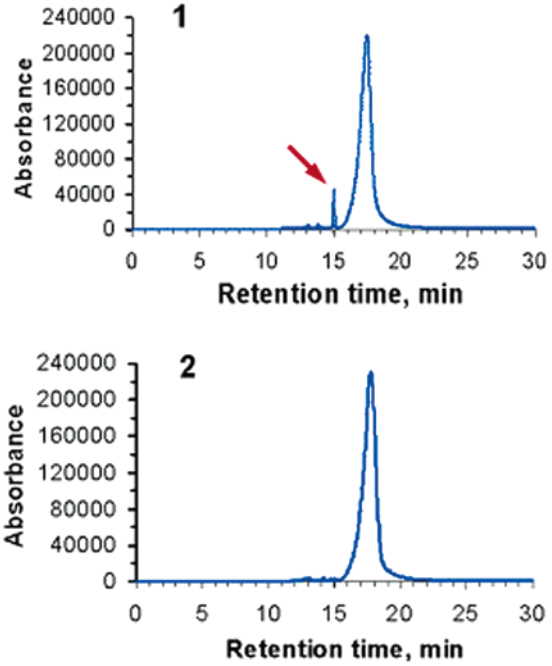


Figure 3. HPLC eluogram of the G5-Ac(96)-FA(5) conjugate (1) before and (2) after membrane filtration purification.

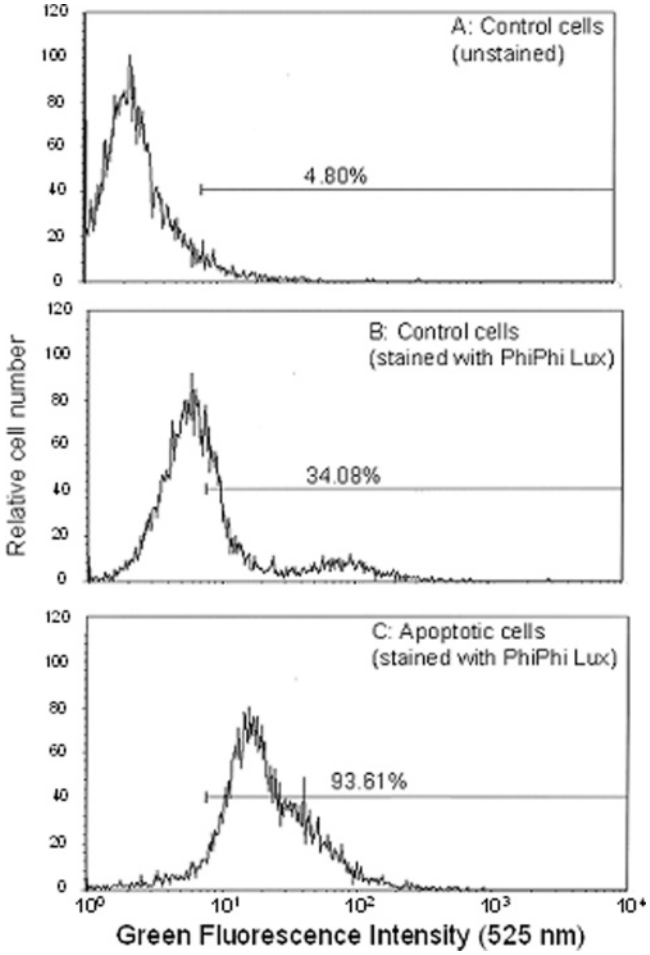


Figure 4. Fluorescence intensity of Jurkat cells stained with PhiPhiLux G_1D_2: (A) background fluorescence of control unstained cells; (B) fluorescence of control stained cells; (C) fluorescence of apoptotic stained cells.

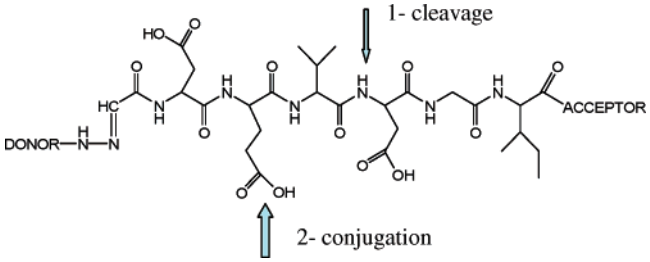


Figure 5. PhiPhiLux G_1D_2 structure: (1) where it is cleaved by caspase-3 enzyme and (2) the carboxyl group participating in the conjugation.

acceptor on PhiPhiLux G_1D_2 conjugated to the dendrimer, thereby increasing the fluorescence intensity in the apoptotic KB cells. Importantly, the conjugation to the polymer prevented internalization into receptor-negative cells.

Significance

The functional studies clearly show that the G5-Ac(96)-FA-PhiPhiLux G_1D_2 macromolecule specifically detects apoptosis in targeted cells (KB cells). The G5-Ac(96)-FA-PhiPhiLux G_1D_2 nanodevice would be the first targeted apoptosis detector developed. Since we have demonstrated that similar molecules can deliver cancer drugs in vivo, this allows the potential for specifically targeting apoptosis detectors to tumors to monitor

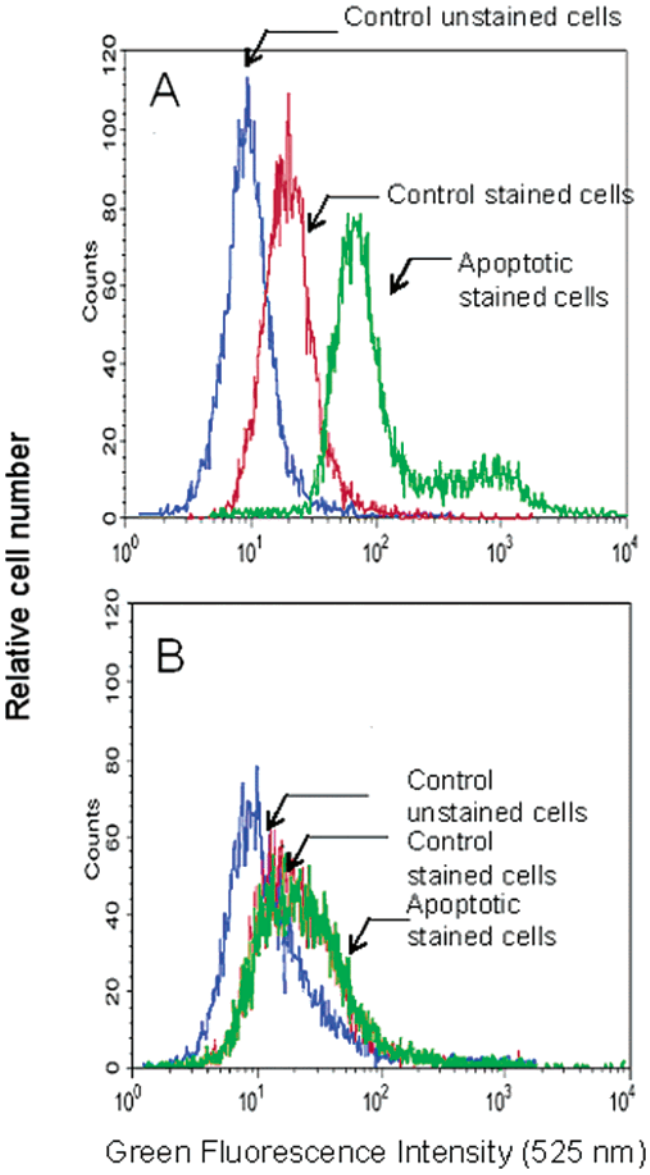


Figure 6. Fluorescence intensities of (A) KB and (B) UMSCC-38 cells treated and untreated with staurosporine and stained with the G5-Ac(96)-FA-PhiPhiLux G_1D_2 nanodevice.

their response to therapy. However, the fluorescence measurement using conventional techniques such as flow cytometry that was used in this study is not applicable for in vivo fluorescence quantification. We have recently made advances in overcoming this problem using a two-photon optical fiber device.[16] In this method an optical fiber is inserted through a 27-gauge needle to quantify the fluorescence of a targeted nanodevice in live mice tumors. We believe systems such as this can be further developed to quantify the changes in FRET-based tissue fluorescence targeted through a carrier such as the dendrimer.

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