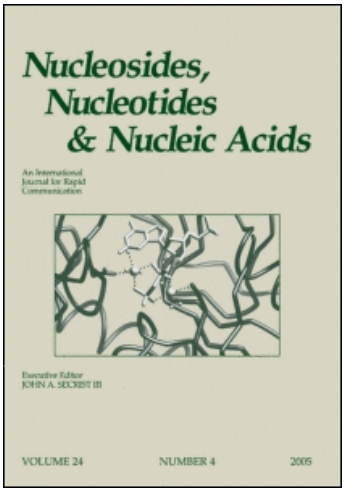

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Synthesis of a Fluorescent Cationic Phosphorus Dendrimer and Preliminary Biological Studies of Its Interaction with DNA

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SYNTHESIS OF A FLUORESCENT CATIONIC PHOSPHORUS DENDRIMER AND PRELIMINARY BIOLOGICAL STUDIES OF ITS INTERACTION WITH DNA

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□ *The synthesis of a water-soluble phosphorus-containing dendrimer possessing a fluorophore (maleimide-type) linked to the core is described. This dendrimer is found brightly fluorescent in CH_2Cl_2, but poorly fluorescent in water. The cytotoxicity of this compound is relatively low towards HeLa and A549 cells, and less toxic after 48 hours than after 24 hours. Association of this dendrimer with plasmid DNA (BACE-GFP) analyzed with circular dichroism (CD) indicates a possible disturbing of the helical B-type structure of DNA. The strength of this association (a "dendriplex") with BACE-GFP (also with HygEGFP) was measured by electrophoresis.*

Keywords Dendrimers; fluorescence; DNA; supramolecular interactions; electrophoresis; circular dichroism

1. INTRODUCTION

Dendrimers[1–3] are nowadays a widely used type of polymer in many areas of the scientific research, including chemistry, materials science, nanotechnologies and physics, biology, and even medicine. Their hyperbranched and chemically perfectly defined structure, as well as their numerous terminal functional groups, certainly explains the keen interest they arouse. One of the distinctive features of these synthetic macromolecules

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is their interaction with "biological entities" in a broad sense due to their multivalency.[4] Multivalent systems are found throughout nature, especially in biology. It is noteworthy that weak ligand-receptor interactions can be made much stronger simply by the simultaneous bonding of ligands to multiple receptors.[5] Concerning dendrimers, their interaction with DNA gave birth to numerous publications.[6] In particular, the utility of polycationic dendrimers as synthetic vectors in transfection experiments has been recognized very early.[7] Many experiments in this field were carried out with Poly(AMidoAMine) dendrimers (PAMAM),[8] but we have also demonstrated that phosphorus-containing dendrimers[9] are usable as vehicles to transport DNA[10,11] or plasmids inside cells.[12]

A mechanism has been proposed for the use of dendrimers as transfecting agents, which implies at all steps supramolecular interactions. The first step is the strong electrostatic association of dendrimers and DNA,[13] affording what has been called a dendriplex.[14] In order to interact with the cell membrane (negatively charged), the dendriplex must be positively charged (an excess of dendrimers is used). Endosomal uptake of this dendriplex allows the internalisation inside the cytoplasm. Dissociation occurs presumably under the influence of cationic lipids. The transfer from the cytoplasm to the nucleus is not fully understood, nor is the behavior of the dendrimer after the transfection. The determination of biological mechanisms often uses fluorescent derivatives, which are very sensitive tools; water-soluble fluorescent dendrimers begin to play an interesting role in this field.[15] Thus, it appeared relevant to graft a fluorescent tag to a type of polycationic dendrimer which was previously found suitable for transfection experiments (phosphorus-containing dendrimers).[12] We have developed earlier several methods to graft various fluorescent entities to dendrimers. The simplest way consists of statistically grafting fluo-tag to the surface; such reaction has been done with fluorescein isothiocyanate (FITC) on an anionic phosphorus-containing dendrimer,[16] but it appeared preferable to use perfectly defined structures. For this purpose, the presence of the fluorescent tag at the core[17,18] or close to the core[19,20] of the dendrimer appears more suitable.

In this article, we describe the synthesis and physicochemical properties of a cationic phosphorus dendrimer bearing the 3,4-diphenylmaleimide fluotag linked to the core, its fluorescence properties depending on the media used, and the preliminary study of its biological properties, in particular its cytotoxicity, and its interaction with plasmid DNA studied by circular dichroism and electrophoresis experiments.

2. RESULTS AND DISCUSSION

We have already reported the synthesis and the fluorescence properties of a series of dendrimers having a 3,4-diphenylmaleimide (fluorescent)

group linked to the core,[21] as well as their potential use as chemical sensor.[22] We could deduce from these previous studies that water induces only a slight decrease of the fluorescence intensity, but that the structure of the dendrimer has a detrimental effect on the fluorescence intensity. Thus, we decided to synthesize only a relatively small cationic dendrimer to try to preserve acceptable fluorescence intensity, but we could foresee that the transfection efficiency of this compound should be low. Indeed we have previously shown that the transfection efficiency of analogous but not fluorescent dendrimers increases from generation 1 to generation 4.[12]

2.1. Synthesis

Our starting compound is the fluorescent second generation dendrimer **1-G$_2$**, which possesses one 3,4-diphenylmaleimide function at the core and 10 P(S)Cl$_2$ terminal functions, prepared as previously described.[21] Reaction of this compound with a stoichiometric amount of N,N-diethylethylene diamine (1 per Cl) affords dendrimers **2-G$_2$** (Figure 1). Hydrogen chloride generated by the reaction is trapped by the terminal nitrogen atoms. Such reaction generates ammonium salts, which induce the solubility of the whole dendritic structure in water. The grafting of the diamine is in particular characterized by ^{31}P NMR, which displays the disappearance of the signal corresponding to R-P(S)Cl$_2$ ($\delta = 62.8$ ppm) on behalf of a new signal corresponding to R-P(S)(NHCH$_2$CH$_2$NEt$_2$,HCl)$_2$ ($\delta = 70.2$ ppm), whereas the signals corresponding to the internal structure remains almost unchanged. The unsymmetrical character of this family of compounds is still detectable at the level of the first generation as shown by ^{31}P NMR, which displays two signals for P$_1$ in a 3:2 ratio at 62.45 and 62.65 ppm, respectively.

2.2. Fluorescence and Physicochemical Studies About Dendrimer 2-G$_2$ Alone

Dendrimer **2-G$_2$** is easily soluble in water, but we thought that it would be important to study its fluorescence properties in various conditions. The fluorescence properties of **2-G$_2$** were first measured in dichloromethane, since this dendrimer is soluble both in some polar organic solvents and in water. Figure 2 displays the fluorescence spectra after excitation at 380 nm; a bright fluorescence is observed at about 510 nm in CH$_2$Cl$_2$. The fluorescence of **2-G$_2$** was then measured in MilliQ water. Deceptively and surprisingly, the fluorescence of this dendrimer in water was very low, using the same concentrations as in CH$_2$Cl$_2$ (see Figure 2). As indicated earlier,[21,22] we have previously shown that the fluorescence of this maleimide group was only slightly affected by water. Thus, we think that the detrimental influence of water is not due to a direct interaction of water with maleimide but is due to the hydrophobicity of the internal structure of the dendrimer, which

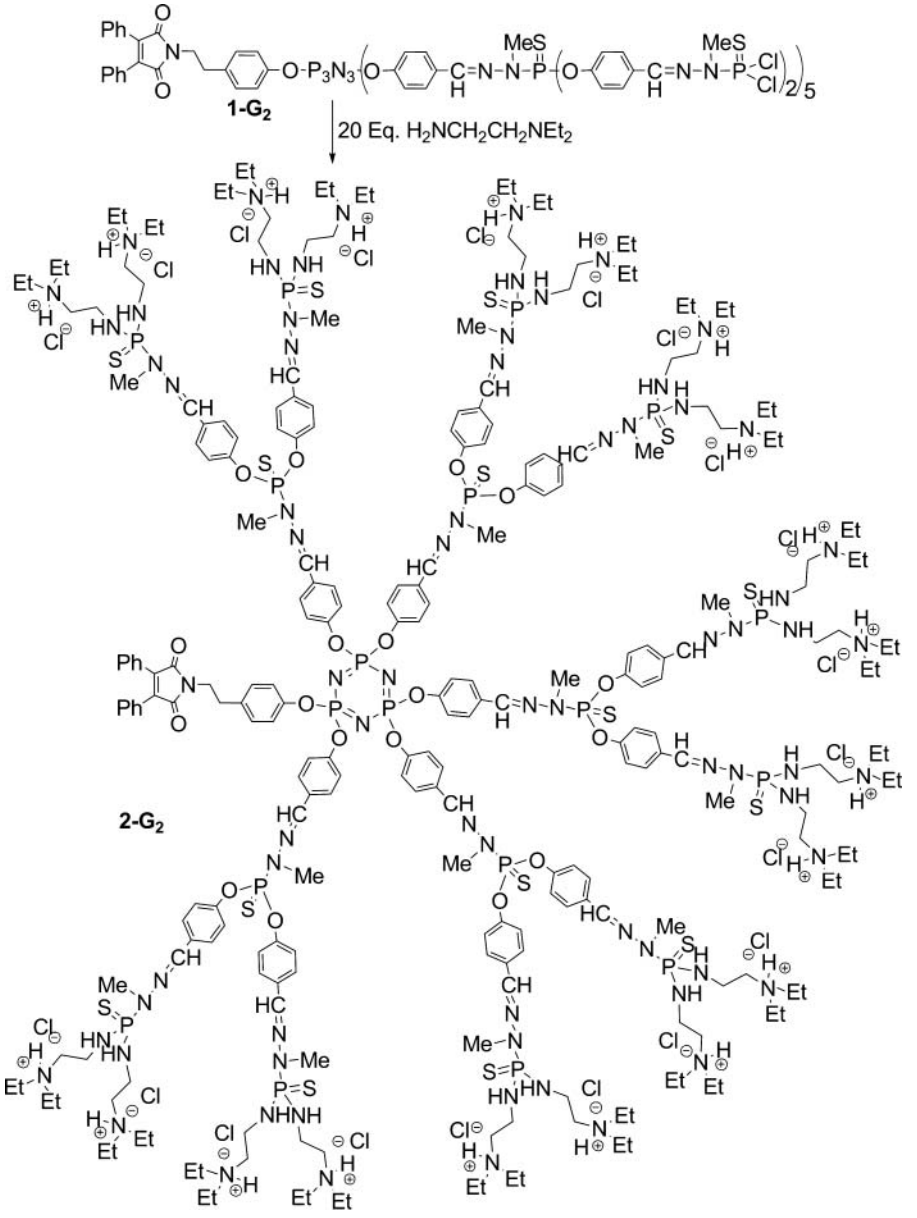

FIGURE 1 Synthesis of the fluorescent cationic dendrimer **2-G$_2$**.

induces geometrical/topological modifications. Indeed, we have previously shown that a generation 5 dendrimer bearing the same ammonium terminal groups is totally shrunk in water, but recovers an expanded structure when adding tetrahydrofuran (THF) to water;[23] the same phenomenon can be anticipated here. As we have also shown that the dendritic branches are able

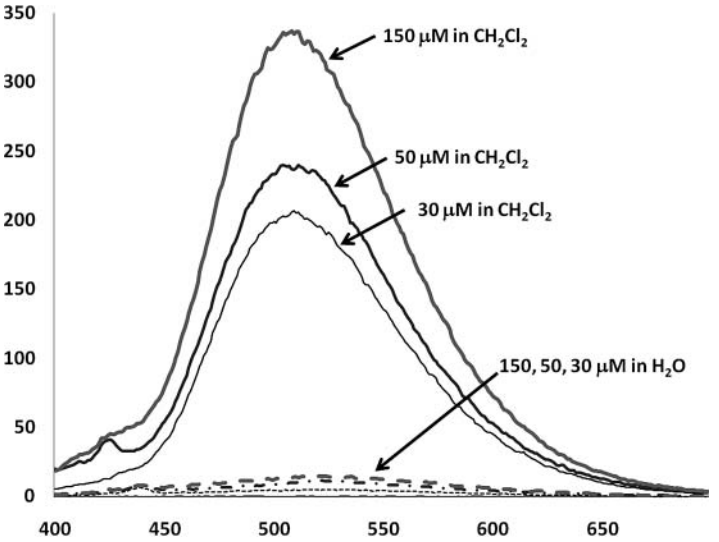

FIGURE 2 Relative fluorescence intensity of dendrimer **2-G$_2$** in CH_2Cl_2 (—) and in MilliQ water with concentrations: 30 (. . . . .), 50 (_ . _ . _ .), and 150 (_ _ _ _) μM of **2-G$_2$**.

to quench the fluorescence of the maleimide group,[21] it is easily understandable that the closer proximity of the branches due to the shrinkage should maximize the quenching effect.

However, the presence of other substances in the media might modify the degree of shrinkage (as THF did previously[23]), and thus might increase the fluorescence intensity. Hence, other experiments were carried out in conditions close to those usable for biological experiments, that is, in phosphate buffered saline (PBS) buffer, in 10 or 20% fetal bovine serum (FBS, which contains proteins, in particular the bovine serum albumin) in RPMI 1640, and in OPTI MEM media (frequently used as cell media). In all cases, the λmax value for the excitation is at 250 nm, and the λmax value for the emission (fluorescence) is at 498 nM, thus the ionic strength has no influence on the maxima wavelength values. Figure 3 displays the fluorescence intensity of dendrimer **2-G$_2$** measured in the various aqueous media described above and at various concentrations, from 0.001 to 1 mM. The fluorescence intensity is slightly increased in PBS and FBS compared to pure water, but it is almost undetectable in OPTI MEM media, excepted at very low concentration. This phenomenon is not due to an additional quenching but to the precipitation of the dendrimer in this media, excepted at very low concentration. Figure 4 shows that dendrimer **2-G$_2$** is very soluble in water even at 10 M, whereas it precipitates almost immediately even at 1 μM in OPTI MEM media. The precipitation occurs more slowly in RPMI 1640 media, but is observable after 48 hours in the most concentrated solutions.

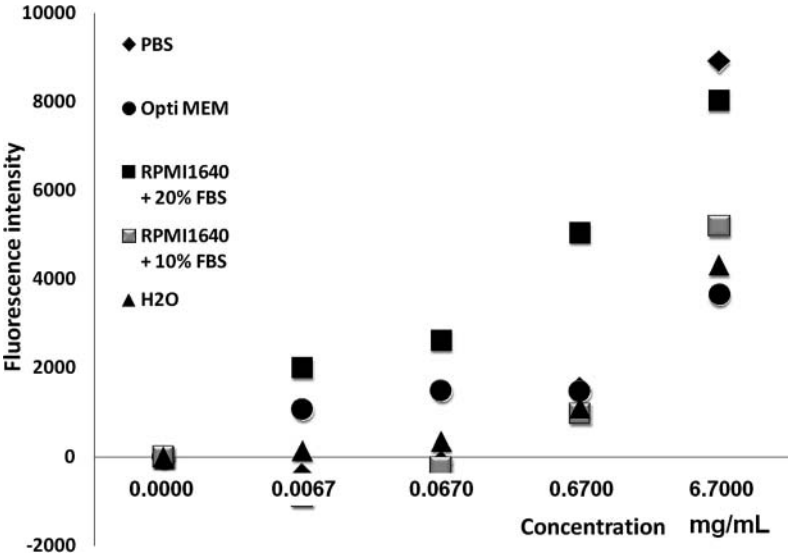

FIGURE 3 Evolution of the fluorescence intensity of dendrimer **2-G$_2$** in various aqueous media (lozenges: PBS; disks: Opti MEM; gray squares: RPMI1640 + 10% FBS; black squares: RPMI1640 + 20% FBS; triangles: MilliQ water) and at different concentrations.

2.3. Influence of Dendrimer 2-G$_2$ on the Cells Viability

After these physicochemical experiments, the next step consists in studying the influence of the presence of this dendrimer on the cell viability. The cytotoxicity of **2-G$_2$** was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This test consists in the reduction of

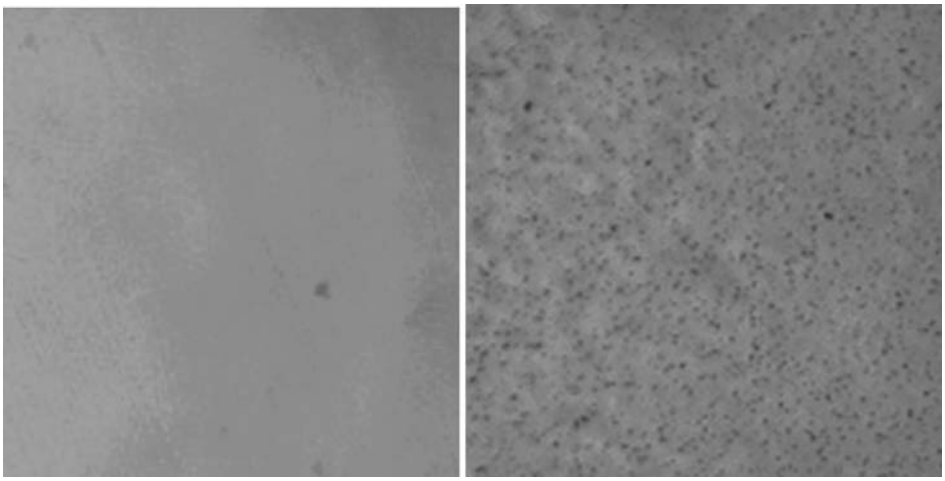

FIGURE 4 Images of dendrimer **2-G$_2$**; left: at 1.0 μM in water; right at 1.0 μM in OPTI MEM media (magnitude 40×).

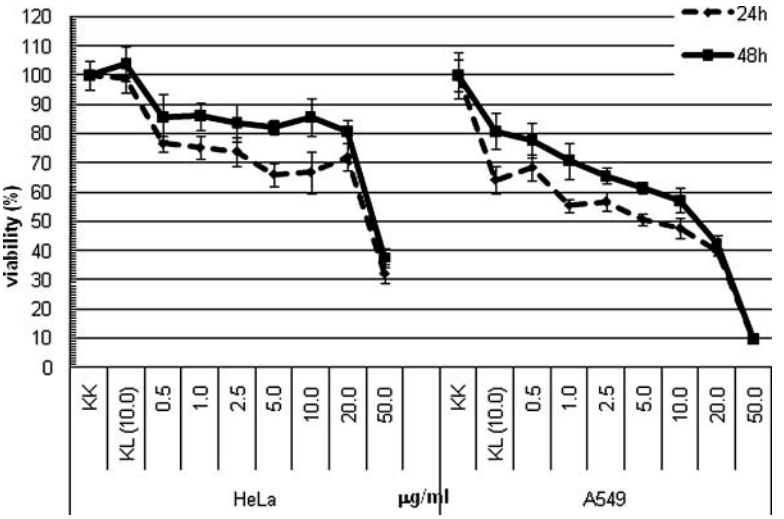

FIGURE 5 Viability of HeLa cells and A549 cells in RPMI 1640 without dendrimer and without lipofectin (KK) with lipofectin (KL, 10 μg/mL) and with various concentrations of dendrimer **2-G$_2$** (from 0.5 to 50 μg/mL) after 24 hours (- - -)and 48 hours (—).

MTT by the succinate dehydrogenase intervening in the respiratory mitochondrial chain of viable cells. The water-soluble yellow MTT is converted to water-insoluble purple formazan, which is later dissolved in organic solvents and dosed by spectrophotometry.[24] The percentage of living cells is deduced from the corrected formazan absorbance. These experiments were carried out with two cells strains HeLa (human epitheloid cervical carcinoma) and A549 (human lung cancer). In both cases two control experiments were carried out, one without dendrimer, another one without dendrimer but with lipofectamine (a common transfection agent) at 10 μg/mL. The dendrimer **2-G$_2$** was used at various concentrations, from 0.5 to 50 μg/mL. All experiments were carried out for 24 hours and for 48 hours, and are shown in Figure 5. It can be deduced form these data that the dendrimer is less cytotoxic towards HeLa cells than towards A549 cells and interestingly, more toxic after 24 hours than after 48 hours. This observation may suggest that tested cells are able to metabolize the dendrimer within the progress of the experiment, and therefore after longer time less agent is present in the cells causing smaller drop of cells viability. The data shown are the mean values of two experiments.

Thus, from all these physicochemical and biological data, it can be deduced that dendrimer **2-G$_2$** should be used preferably at concentrations between 1.0 and 10 μM, without a long incubation. However, the physicochemical data (in particular the solubility, and may be the fluorescence also) should be very different for the dendrimer in interaction with DNA; this was the next series of experiments we attempted.

2.4. Supramolecular Interactions Between Dendrimer 2-G_2 and Plasmid DNA

The interaction of dendrimer **2-G_2** with DNA was more precisely carried out with the plasmid DNA coding the gene of fluorescent fusion protein BACE-GFP, in OPTI MEM media. Structure of the association was evaluated first by circular dichroism (CD) spectroscopy. CD is undoubtedly one of the most powerful techniques for investigating the modifications within the DNA secondary structure induced in particular by ligand binding, even if spectral changes cannot be interpreted on a quantitative theoretical base. The CD spectra were recorded in ultraviolet. The intrinsic CD activity of the dendrimer alone was found negligible, whereas DNA alone exhibits CD spectrum with a positive band at about 277 nm and a negative band at about 243 nm. Figure 6 shows the observed effects on the CD spectra produced by addition of an increasing quantity of dendrimer **2-G_2** to DNA (2:1 to 8:1 ratio of dendrimer terminal groups to bases of DNA). A red shift of the isoeliptic point from 259 to 267 nm was observed, as well as a decrease of the intensity of both the positive and the negative band on complexation with the dendrimer, which might be due to the partial dendriplex precipitation. Relatively analogous CD spectral changes were previously observed for DNA complexed with positively charged PAMAM[25,26] and PPI[27] dendrimers, and are indicative of a possible partial disturbing of the helical B-type structure of DNA.

Another way to characterize the complexation of DNA consists in measuring the strength of the association dendrimer/DNA by electrophoresis. This technique allows us to separate species on a gel support, depending on

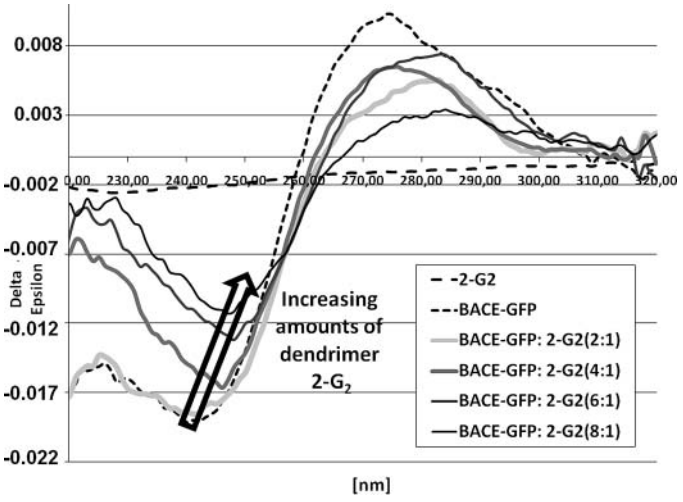

FIGURE 6 Evolution of the circular dichroism (CD) curves of DNA in the presence of dendrimer **2-G_2**. The ratios indicated concern terminal groups of dendrimer to bases of DNA.

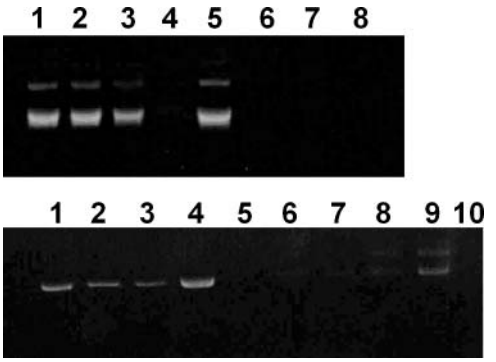

FIGURE 7 Electrophoresis experiments. Up: BACE GFP plasmid (lane 1: BACE GFP alone in OPTI MEM medium; lanes 2–4: complex of plasmid and dendrimer **2-G$_2$** in ratio 4:1, 6:1, 8:1 (terminal groups of **2-G$_2$**/bases of BACE GFP), respectively, in OPTI MEM medium; lane 5: BACE GFP alone in RPMI 1640 medium; lanes 6–8: complex of plasmid and dendrimer **2-G$_2$** in ratio 4:1, 6:1, 8:1 (terminal groups of **2-G$_2$**/bases of BACE GFP), respectively, in RPMI 1640 medium). Down: HygEGFP plasmid (lanes 1–3: complex of plasmid and dendrimer **2-G$_2$** in ratio 4:1, 6:1, 8:1 (terminal groups of **2-G$_2$**/bases of HygEGFP), respectively, in OPTI MEM medium; lane 4: HygEGFP alone in OPTI MEM medium; lane 5: dendrimer **2-G$_2$** alone in OPTI MEM medium; lanes 6–8: complex of plasmid and dendrimer **2-G$_2$** in ratio 4:1, 6:1, 8:1 (terminal groups of **2-G$_2$**/bases of HygEGFP), respectively, in RPMI 1640 medium; lane 9: HygEGFP alone in RPMI 1640 medium; lane 10: dendrimer **2-G$_2$** alone in RPMI 1640 medium).

their charge and molecular mass: Free DNA (negatively charged) should migrate toward the anode faster than DNA complexed with positively charged dendrimers, since most charges should be neutralized in this high molecular mass complex. Figure 7 displays the results obtained with two types of plasmids (BACE GFP and HygEGFP), in two types of media (OPTI MEM and RPMI 1640), and for various ratios of terminal groups of **2-G$_2$**/anions of plasmids (4:1; 6:1; 8:1; it is calculated that negative charges of 1 μg of DNA are equal to positive charges of 1 μg of dendrimer). In the case of the BACE GFP plasmid, only the 8:1 ratio efficiently screens the negative charges in OPTI MEM medium, whereas the 4:1 ratio is sufficient in RPMI 1640 medium. In the case of the HygEGFP plasmid, even the 8:1 ratio is not sufficient in OPTI MEM medium, whereas all ratios screen the charges in RPMI 1640 medium. In fact these results are mainly correlated to the solubility of the dendrimer alone in the medium, as shown in Figure 4: when the dendrimer precipitates (in OPTI MEM media), it cannot efficiently bind to DNA.

3. CONCLUSION

We have synthesized a new dendritic tool, designed to possess both ammonium functions suitable for inducing solubility in water and for interacting with DNA, and a fluorescent tag precisely placed close to the core to minimize its interaction with the biological processes. Measurements of

the fluorescence intensity of this dendrimer in CH_2Cl_2 give a bright fluorescence, whereas measurements in various aqueous media and at various concentrations give weak fluorescence intensity at the same concentration. The cytotoxicity of this compound measured towards two cancerous cell strains (HeLa and A549) indicates a relatively good tolerance of the cells after 24 hours (up to 10 μg/mL) and a surprising cytotoxicity decrease after 48 hours. These preliminary data gave us the range of concentrations usable for studying the interaction of this dendrimer with DNA. Circular dichroism spectroscopy of the interaction dendrimer/plasmid DNA in various ratios indicates a partial disturbing of the helical B-type structure of DNA, and electrophoresis experiments confirm the interaction, and thus the occurrence of a "dendriplex" in several cases. The relatively low cytotoxicity and the interaction with two types of plasmid DNA indicate that this dendritic tool is potentially interesting for transfection experiments, but the low fluorescence intensity indicates that it is not suitable to monitor the biological events associated with transfection. Thus, we envisage the choice of a fluorophore having a more intense fluorescence, and a larger generation dendrimer (higher density of charges) for improving the transfection efficiency and its monitoring.

4. EXPERIMENTAL

4.1. Synthesis of the Dendrimer 2-G_2

To a solution of dendrimer **1-G_2** (235 mg, 53.8 μmol) in 35 mL of dry THF, was added dropwise N,N-diethylethylenediamine (160 μL, 1.13 mmol). The reaction was stirred at room temperature overnight. The solvent was then removed by filtration and the resulting product was washed twice with THF to afford the dendrimer **2-G_2** as a yellow powder in 91.7% yield.

$^{31}P-\{^1H\}$ NMR (CD_3OD, 121.49 MHz): $\delta = 9.14$ (P=N), 62.45 (P_1=S), 62.65 (P_1=S), 70.21 (P_2=S). ^1H NMR (CD_3OD, 300 MHz): $\delta = 1.30$ (m, 120H, $CH_2-\underline{CH_3}$), 2.90 (m, 2H, C'^a-H), 3.05–3.50 (m, 207H, CH_3-N-P_1, CH_3-N-P_2, $\overline{CH_2}$, C'^b-H), 6.80–7.10 (m, 14H, $C'_0{}^2-H$, $C_0{}^2-H$, $C'_0{}^3-H$), 7.15–7.30 (d, 20H, $C_1{}^2-H$), 7.30–7.45 (s, 10H, $C^\circ-H$, C^m-H, C^p-H), 7.50–7.90 (m, 45H, $C_0{}^3-H$, $C_1{}^3-H$, CH=N).

4.2. Study of the Biological Properties of the Dendrimer 2-G_2

4.2.1. Fluorescence Measurements

Fluorescence measurements were made in 1 cm cuvettes using fluorescence spectrophotometer Cary Eclipse (Varian Australia, Clayton, South Australia). Samples at different concentrations of dendrimer **2-G_2** were dissolved in CH_2Cl_2 and in tested aqueous media (phosphate saline buffer (PBS), cell culture media Opti MEM, RPMI1640 + 10% FBS (foetal bovine

serum), RPMI1640 + 20% FBS and deionized water (MilliQ)). Spectra were recorded in triplicate at 25°C. The excitation and emission slits were set at 5 nm.

4.2.2. Microscopic Analysis

Microscopic analysis was performed under epifluorescence, with a phase-contrast Optiphot-2 microscope (Nikon, Japan), using UV-2A filter (Ex 330–380 nm). The DXM 1200 camera equipped with Lucia G 4.61 program was employed for documentation of the dendrimer precipitation process.

4.2.3. Cytotoxicity Experiments

The cytotoxicity of dendrimers was determined in HeLa and A549 cell lines using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide] assay. After 24 hours of cell plating dendrimer was added to a final concentration: 0.5, 1, 2.5, 5, 10, 20, or 50 μg/ml. Lipofectamine was used for control experiments at a final concentration 10 μg/ml (commonly used in experiments). Treated cells were incubated 24 or 48 hours. As a control, cultured cells were grown in the absence of dendrimers or lipofectamine. After incubation 25 μl of the MTT solution (5 mg/ml) was added to each well and incubated for 2 hours. Finally, 95 μl of the lysis buffer (20% SDS, 50% aqueous dimethylformamide, pH 4.5) was added and incubation was carried out for additional 24 hours. Absorbance of sample was measured at 570 nm, with the reference wavelength of 630 nm. The percentage of living cells (PLC) was obtained from the following equation: PLC = (AS – AM)/(AC – AM) × 100%, where AS corresponds to the sample treated by the dendrimer, AM is the absorbance due to the diffusion media, and AC is the absorbance of a sample reference in the absence of the dendrimer. Data points represent means of at least eight repeats.

4.2.4. Circular Dichroism Measurements

In order to determine CD spectra, the plasmid DNA coding the gene of fluorescent fusion protein BACE-GFP (2 M solution) dissolved in OPTI MEM medium was treated with dendrimer solution to obtain the plasmid DNA/dendrimer molar ratio from 1/1 to 1/8. The CD spectra were recorded on a CD6 dichrograph (Jobin-Yvon, Longjumeau, France) using cuvettes with 0.5 cm path length, 2 nm bandwidth, and 1–2 seconds integration time. Each spectrum was smoothed with a 25-point algorithm (included in the manufacturer's software, version 2.2.1). The spectra in range from 200 nm to 350 nm were recorded at 25°C.

4.2.5. Electrophoresis Experiments

OPTI MEM and RPMI1640 solutions of BACE GFP (0.1 μg/μl) and HygEGFP (0.22 μg/μl) plasmid DNAs and solutions of the **2-G$_2$** dendrimer

in the same media (1 μg/μl) were incubated for 5 minutes at room temperature and mixed to give mixtures of the plasmids to dendrimer ratio of **1:4**, **1:6**, **1:8**. These complexes were incubated for 20- 40 minutes at room temperature and amounts containing 0.5 μg of plasmid DNA were diluted with 2 μl of dye (bromophenol blue/ saccharose). Electrophoresis was performed in 0.5% agarose gel and in 90V voltage. The gel was visualized by ultraviolet light and photographic documentation.

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