Design of a New Multifunctionalized PAMAM Dendrimer with Hydrazide-Terminated Spacer Arm Suitable for Metal−Carbonyl Multilabeling of Aldehyde-Containing Molecules

Nathalie Fischer-Durand,[†] Michèle Salmain,[†] Bogna Rudolf,[‡] Lauriane Jugé,[§] Vincent Guérineau,[||] Olivier Laprévote,[||] Anne Vessières,*[,†] and Gérard Jaouen[†]

Ecole Nationale Supérieure de Chimie de Paris (ENSCP), Laboratoire de Chimie et Biochimie des Complexes Moléculaires CNRS UMR 7576, 11 rue P. et M. Curie, 75231 Paris cedex 05, France, University of Lodz, Department of Organic Chemistry, 90-136 Lodz, Narutowicza 68, Poland, ENSCP, Service de RMN, 11 rue P. et M. Curie, 75231 Paris cedex 05, France, and Institut de Chimie des Substances Naturelles (ICSN), UPR-2301 CNRS, Laboratoire de Spectrométrie de Masse, Av. de la Terrasse, 91198 Gif sur Yvette Cedex, France

Received July 20, 2007; Revised Manuscript Received September 17, 2007

ABSTRACT: We describe herein the synthesis of generation 4 (G4) poly(amidoamine) (PAMAM) dendrimers bearing (1) a nitroaniline derivative as an NMR probe, (2) (η^5-cyclopentadienyl)iron dicarbonyl succinimidato complexes as infrared probes for carbonyl metallo immunoassay, (3) acetamide groups for increased solubility, and (4) hydrazide functions at the extremity of five-carbon atom spacer arms for the labeling of aldehyde-containing molecules. The step-by-step building of these multifunctionalized dendrimers was monitored by ^{1}H NMR spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, and by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Substitution of terminal amine groups of PAMAM dendrimers with appropriate functional groups provides complex dendrimeric structures with a wide range of applications. The final goal of this study is the development of new reagents suitable for the metal−carbonyl multilabeling of immunological reagents such as antibodies.

Introduction

Poly(amidoamine) (PAMAM) dendrimers are new synthetic polymers with well-defined molecular structures and polydispersities close to unity.[1−3] They are constructed from an amine core (ammonia, ethylenediamine, cystamine) through alternate reactions of methyl acrylate and ethylenediamine to produce generation 0 to generation 10 (G0−G10) PAMAM dendrimers, corresponding to each two-step reaction sequence, with amine surface functionalities that double at each generation. Their water solubility, combined with their lack of immunogenicity, has led to a wide range of biomedical applications by functionalization of their peripheral groups with various compounds, such as targeting, therapeutic, and diagnostic agents, alone or in combination.[4−11] Their use in the field of biosensors has also been investigated, for instance, as building blocks for the design of biosensing interfaces that favor ligand functionalization and biospecific interaction.[12−17] Dendrimers functionalized with fluorescent probes have also found application in the determination of environmental contaminants.[18−20]

In our laboratory, we have been studying for some time the potential use of dendrimers as carriers of transition-metal−carbonyl complexes, with the objective of developing new generations of universal immunodetection reagents. This research is aimed at the amplification, by multiple labeling, of the infrared detection signal in the carbonyl metallo immunoassay (CMIA) we have developed since the early 1990s.[21−24] For this purpose, we have recently described the preparation of a bifunctional G4-PAMAM dendrimer bearing 45 (η^5-cyclopentadienyl)iron dicarbonyl succinimidato (Fp) units and four biotin entities,[25] and the synthesis of G3- and G4-PAMAM dendrimers labeled with (η^5-cyclopentadienyl)rhenium tricarbonyl [CpRe(CO)$_3$] units with or without attachment of polyethylene glycol (PEG) moieties of various chain lengths.[26] We have also reported the conjugation of G4-PAMAM bearing 10−25 Fp units to the glycosylated part of secondary antibodies.[27] In this latter report, we showed that direct conjugation of an amine-terminated dendrimer to periodate-oxidized immunoglobulin G (IgG) through reductive amination afforded immunoconjugates with a metal−carbonyl dendrimer to IgG ratio that was related to the steric hindrance around the remaining amino groups on the surface of the dendrimer. For instance, with a dendrimer carrying 10 Fp complexes, we were able to conjugate 1.4 dendrimers per IgG, whereas the use of a dendrimer carrying 25 Fp complexes led to the attachment of only 0.5 dendrimer per IgG. These results prompted us to engineer a more advanced generation of metallocarbonyl PAMAM dendrimers bearing a spacer arm so as to move the dendrimer away from the IgG, thus decreasing steric hindrance between the two macromolecules. The same anchoring site for the antibody molecule will be used via oxidation of the glycosylated moieties.

Here we describe the design, synthesis, and characterization of a hydrazide-based trifunctional G4-PAMAM dendrimer bearing Fp complexes (Scheme 1) intended to be used for the labeling of aldehyde-containing molecules, such as oxidized antibodies. Characterization of the dendrimer after the successive functionalization steps was achieved by combining several spectroscopic techniques, including ^{1}H NMR, UV−vis, FT-IR, and MALDI-TOF mass spectrometry.

---

* Corresponding author. E-mail: a-vessieres@enscp.fr.

[†] Laboratoire de Chimie et Biochimie des Complexes Moléculaires, ENSCP.

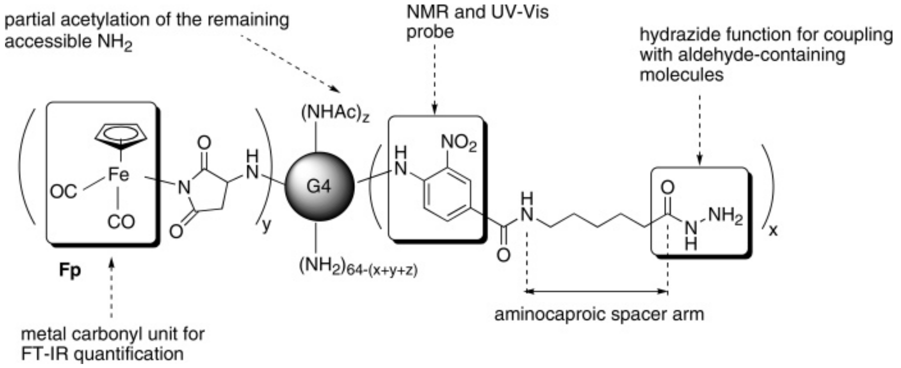
[‡] University of Lodz.

[§] Service de RMN, ENSCP.

[||] ICSN.

10.1021/ma071621g CCC: $37.00

Scheme 1. Design of a New Metal−Carbonyl-Labeled G4-PAMAM Dendrimer



Experimental Section

Materials. G4-PAMAM dendrimer, 4-fluoro-3-nitrobenzoic acid, *N*,*N*-diisopropylethylamine (DIPEA), acetic anhydride, *tert*-butyl carbazate, 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl), trifluoroethanol, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU), ϵ-amino-*n*-caproic acid (aminocaproic acid), and 2,4,6-trihydroxyacetophenone (THAP, used as the matrix for MALDI-TOF experiments, was of the highest grade available and used without further purification) were purchased from Sigma-Aldrich Co. Cytochrome *c*, horse heart myoglobin, and trypsinogen were from LaserBio Labs (LBL, France). Piperazinomethylpolystyrene resin was from Novabiochem. Fp-maleimide was synthesized as described previously.[28] Dialysis was performed with Spectra/Por 1 membranes with a molecular weight cutoff of 6−8000 Da (from Spectrum Laboratories, Inc.). Nitrocellulose membranes (pore size, 0.45 μm) were purchased from Bio-Rad (product no. 162−0117). Flash chromatography was performed on silica gel 60 (Merck, 40−63 μm). UV/vis spectra were recorded on a UV/mc^2 spectrometer (Safas). NMR spectra were recorded on BRUKER Avance 300 and BRUKER Avance 400 spectrometers; 10 mM NaPB pH 7.2 buffer contained 0.2 M NaH_2PO_4 (11 mL) and 0.2 M Na_2HPO_4 (39 mL) diluted to 1 L of deionized water with 0.15 M NaCl.

FT-IR Spectroscopy. FT-IR spectra were recorded on a benchtop MB 100 spectrometer (Bomem) equipped with a liquid nitrogen-cooled MCT detector and a 6 mm-diameter membrane holder perpendicularly positioned with respect to the IR beam. FT-IR data were recorded and manipulated on a Windows-operating PC using the WinBomemEasy software. Routinely, 44 scans were coadded in about 1 min, and the resulting interferogram was apodized using a cosine function and then Fourier-transformed to yield a 4 cm^{-1} resolution spectrum. The baseline corrected absorbances were measured using the Quant method included in the software.

The calibration curve used for the quantification of the metallodendrimers was established as follows: standard solutions of Fp-maleimide in the range 0.66−0.05 μmol/mL in 10 mM NaPB pH 7.2 were spotted (4 μL) onto punched nitrocellulose membranes (diameter = 6 mm). A blank was made by spotting 4 μL of NaPB buffer. Membranes were dried for at least 2 h at room temperature before IR recording in the range 1800−2200 cm^{-1}. The calibration curve was constructed by plotting absorbance at 2052 cm^{-1} versus concentration of the standard solutions of Fp-maleimide. This experiment was repeated several times and gave reproducible standard straight lines.

Quantification of the Fp entity in the metallodendrimer solutions was carried out as follows: the conjugate was put in 2 mL of MeOH and then diluted in 10 mM NaPB buffer pH 7.2 (two dilutions were made between dilutions 20 and 80). Four microliters in duplicate were spotted onto nitrocellulose membranes and dried. A blank was made by spotting 4 μL of NaPB buffer. FT-IR spectra were then recorded between 1800 and 2200 cm^{-1}. Absorbances at 2052 cm^{-1} were used to calculate the average concentration of Fp entities using the calibration curve established previously.

MALDI-TOF Mass Spectrometry. A Voyager DE-STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA), equipped with a 337-nm pulsed nitrogen laser (20 Hz) and an Acqiris 2 GHz digitizer board, was used for all experiments.

Mass spectra were obtained in linear positive ion mode with the following settings: accelerating voltage of 25 kV, grid voltage 94% of accelerating voltage, extraction delay time of 700 ns. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (*S*/*N*) ratio without significant peak broadening.

The mass spectrometer was externally calibrated using the protonated molecule of cytochrome *c* ($[M + H]^+$, *m*/*z* 12 361.12), horse heart myoglobin ($[M + H]^+$, *m*/*z* 16 952.50), and trypsinogen ($[M + H]^+$, *m*/*z* 23 982.00).All data were processed using the Data Explorer software package (Applied Biosystems).

2,4,6-Trihydroxyacetophenone (THAP) was used as the matrix. The THAP matrix solution was prepared by dissolving 10 mg in 500 μL of water and 500 μL of methanol. One microliter of the analyte ([G4]= 98 μM) and 9 μL of the matrix solution were mixed together, and 1 μL of the resulting solution was spotted on the MALDI plate and air-dried ("dried droplet" method).

Precautions for Handling Dendrimers. Functionalized dendrimers must be kept in solution at all times, otherwise it is not possible to dissolve them again. Consequently, compounds cannot be thoroughly dried before NMR recording. Accordingly, to eliminate MeOH traces, each sample is evaporated to dryness for no more than 1 min and then dissolved two times with 0.8 mL of MeOH-d_4 and evaporated.

Synthesis of PAMAM G4−Ar$_5$ (Conjugate 1). G4-PAMAM (400 μL, 2.29 μmol, 10% solution in methanol) was added to a solution of 4-fluoro-3-nitrobenzoic acid (2.12 mg, 5 equiv) and DIPEA (4 μL, 10 equiv) in methanol (1.2 mL). The solution was stirred at room temperature for 48 h and then transferred into a dialysis membrane and extensively dialyzed in methanol to remove unbound 4-fluoro-3-nitrobenzoic acid, ammonium salt, and excess DIPEA until the dialysis solvent no longer absorbed in the 200−500 nm range. The dialyzed sample was then evaporated to dryness for no more than 1 min, dissolved two times with 0.8 mL of MeOH-d_4, and evaporated (see Precautions for Handling Dendrimers). The ^{1}H NMR spectrum was recorded in MeOH-d_4 on a 300 MHz NMR spectrophotometer, and the peaks of the three aromatic protons of the nitrobenzoic derivative were characterized (Figure 1, spectrum A). The average number of nitrobenzoic acid derivatives attached per dendrimer was calculated by dividing the integral value of one aromatic proton with the integral value of the 248 protons at 2.3 ppm normalized to one proton (see Results and Discussion). In addition, conjugate **1** was analyzed by UV−vis spectroscopy at 428 nm to quantify the chemically bound nitrobenzoic derivative and, further, the dendrimer. For this purpose, a calibration curve was established using known concentrations of 4-butylamino-3-nitrobenzoic acid in methanol (compound **7**). Conjugate **1**, termed G4−Ar$_5$, was obtained in 80% yield and stored in methanol at −20 °C.

Synthesis of PAMAM G4−Ar$_5$Fp$_y$ (Conjugate 2). Fp-maleimide (20.1 mg, 40 equiv) was added to a solution of G4−Ar$_5$ (1.84 μmol) in MeOH (2 mL). The solution was stirred at room temperature in the dark for 3−4 days and then transferred into a

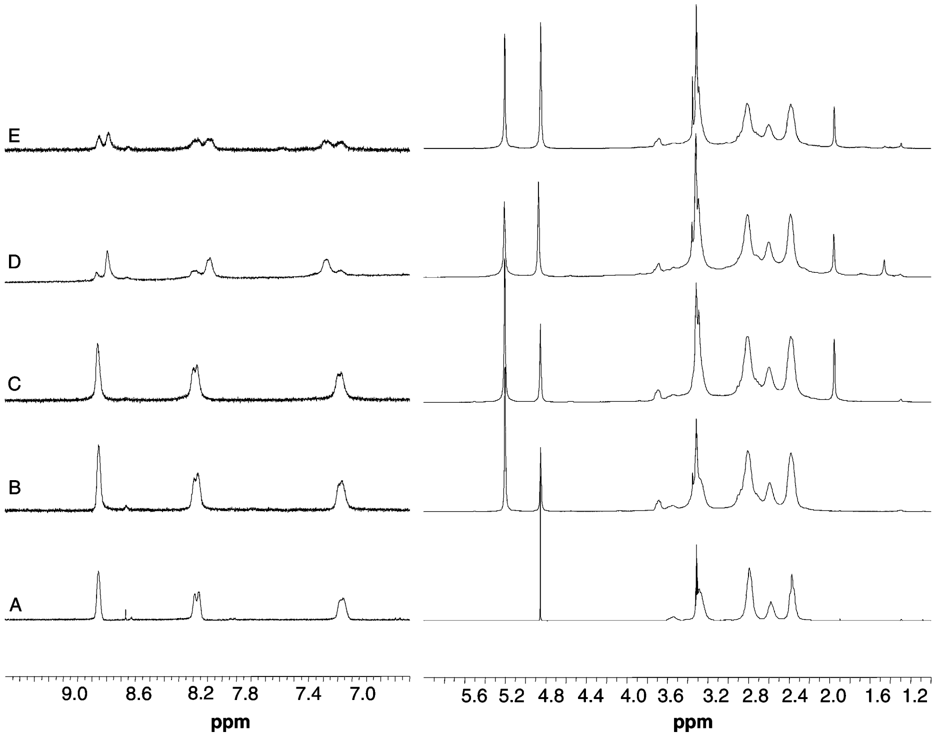


Figure 1. ^{1}H NMR spectra of G4$-$Ar$_x$ (**1**) (A), G4$-$Ar$_x$Fp$_y$ (**2**) (B), G4$-$Ar$_x$Fp$_y$NHAc$_z$ (**3**) (C), G4$-$Fp$_y$NHAc$_z$(ArHydr-NHBoc)$_{x'}$ (**5**) (D) and G4$-$Fp$_y$NHAc$_z$(ArHydr-NH$_2$)$_{x'}$ (**6**) (E).

dialysis membrane and extensively dialyzed in methanol to remove unbound Fp-maleimide until the dialysis solvent no longer absorbed in the 200$-$500 nm range (4$-$5 days). The solution was then evaporated as described for conjugate **1** and characterized by ^{1}H NMR in MeOH-d_4 (Figure 1, spectrum B). The average number of Fp complexes attached per dendrimer was calculated by multiplying the integral value of the Cp protons signal at 5.2 ppm normalized to one proton by the number of nitrobenzoic derivatives determined at the previous step, i.e., five (the integral value of the peak at 8.76 ppm related to one proton of the nitrobenzoic ring is calibrated to 1 throughout this study). The average extent of conjugation of 25$-$32 Fp complexes per dendrimer was found after several experiments. In addition, conjugate **2** was analyzed by FT-IR spectroscopy to quantify the concentration of Fp entities according to the procedure described in the FT-IR Spectroscopy section, yielding the concentration of the dendrimer. Conjugate **2**, termed G4$-$Ar$_5$Fp$_y$, was obtained in 80% yield and stored in methanol at -20 °C.

Synthesis of PAMAM G4$-$Ar$_5$Fp$_y$NHAc$_z$ (Conjugate 3). MeOH was evaporated from conjugate **2** solution (1.47 μmol). CF$_3$CH$_2$OH (1 mL) was quickly added and then evaporated (twice). CF$_3$CH$_2$OH (1.5 mL) was added followed by DIPEA (22.5 μL, 10% excess based on the amount of acetic anhydride) and then acetic anhydride (11.1 μL, 80 equiv/G4). The solution was stirred at room temperature in the dark for 24 h, transferred into a dialysis membrane, and dialyzed in MeOH/H$_2$O (1:1) for at least 48 h and then in MeOH for 48 h. The solution was then evaporated as described for conjugate **1** and characterized by ^{1}H NMR in MeOH-d_4 (Figure 1, spectrum C). The average numbers of Fp complexes and acetyl groups attached per dendrimer were calculated from the integral values in the same manner as described for conjugate **2**. The average extent of conjugation of 25$-$30 acetyl groups per dendrimer was found after several experiments. Furthermore, conjugate **3** was analyzed by FT-IR spectroscopy to quantify the concentration of Fp entities according to the procedure described in the FT-IR Spectroscopy section, yielding the concentration of dendrimer. Conjugate **3**, termed G4$-$Ar$_5$Fp$_y$NHAc$_z$, was obtained in 75% yield and stored in methanol at -20 °C.

Synthesis of Hydrazine Carboxylic Acid, 2-(6-Amino-1-oxohexyl)-1-*tert*-butyl Ester 4. FmocCl (0.627 g, 2.35 mmol) in dioxane (5 mL) was added dropwise to a solution of aminocaproic acid (0.3 g, 2.286 mmol) in 10% Na$_2$CO$_3$ solution (10 mL). The mixture was stirred at room temperature for 36 h. H$_2$O was then added (70 mL) and the solution was acidified with concentrated HCl. The precipitate was filtered off, rinsed with slightly acidic water, and then dissolved in chloroform and dried (Na$_2$SO$_4$). The crude acid was purified by flash chromatography on silica gel (EtOAc/CH$_2$Cl$_2$, 50:50 to remove apolar compounds then 80:20) to afford the intermediate FmocNH(CH$_2$)$_5$CO$_2$H as a white powder (0.74 g, 91% yield): mp 114 °C; ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.32 (m, 2 H), 1.45 (m, 2 H), 1.55 (m, 2 H), 2.27 (t, $J = 7.3$ Hz, 2 H), 3.11 (q, $J = 6.6$ Hz, 2 H), 4.11 (t, $J = 6.6$ Hz, 1 H), 4.32 (d, $J = 6.6$ Hz, 2 H), 4.72 (br s, NH), 7.23 (t, $J = 7.2$ Hz, 2 H), 7.31 (t, $J = 7.2$ Hz, 2 H), 7.51 (d, $J = 7.2$ Hz, 2 H), 7.68 (d, $J = 7.2$ Hz, 2 H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 23.2, 26.1, 29.6, 33.7, 40.8, 47.3, 66.5, 119.9, 125.0, 127.0, 127.6, 141.3, 144.0, 156.4, 178.8.

DIPEA (177 μL, 1.02 mmol) and TSTU (0.281 g, 0.935 mmol) were added to a solution of FmocNH(CH$_2$)$_5$CO$_2$H (0.3 g, 0.85 mmol) in dioxane (5.5 mL). The solution was stirred at room temperature for 1 h, *tert*-butylcarbazate was added (0.135 g, 1.02 mmol), and the solution was stirred for 48 h. EtOAc (40 mL) and H$_2$O (30 mL) were added, and the aqueous layer was extracted with EtOAc (3 × 40 mL) and dried (Na$_2$SO$_4$). The crude hydrazide was purified by flash chromatography on silica gel (EtOAc/CH$_2$Cl$_2$, 70/30) to afford the intermediate FmocNH(CH$_2$)$_5$CONHNHBoc as a colorless oil (0.28 g, 72% yield): ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.28 (m, 2 H), 1.36 (s, 9 H), 1.44 (m, 2 H), 1.58 (m, 2 H), 2.12 (t, $J = 7.1$ Hz, 2 H), 3.08 (q, $J = 6.2$ Hz, 2 H), 4.11 (t, $J = 6.8$ Hz, 1 H), 4.29 (d, $J = 6.8$ Hz, 2 H), 5.07 (br s, NH), 6.71 (br s, NH), 7.21 (t, $J = 7.5$ Hz, 2 H), 7.30 (t, $J = 7.5$ Hz, 2 H), 7.50 (d, $J = 7.2$ Hz, 2 H), 7.66 (d, $J = 7.5$ Hz, 2 H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 24.7, 26.0, 28.1, 29.4, 33.7, 40.7, 47.3, 66.5, 81.7, 119.9, 125.0, 127.0, 127.6, 141.3, 144.0, 155.8, 156.6, 172.7; *m*/*z* (CI/NH$_3$) 485 (20%, [M + NH$_4$]$^+$), 468 (30%, [M + H]$^+$), 429 (15), 412 (15), 368 (52), 246 (50), 179 (100); HRMS (CI/CH$_4$) *m*/*z* calcd for C$_{26}$H$_{34}$O$_5$N$_3$ (M + H)$^+$ 468.2498, found 468.2508.

Removal of the Fmoc Protecting Group. The piperazinyl resin (2.3 g, 0.86 mmol g^{-1}) was allowed to swell with THF/H$_2$O (12 mL, 3:1) under gentle stirring at room temperature for 48 h, and then $FmocNH(CH_2)_5CONHNHBoc$ (0.15 g, 0.329 mmol) in THF (0.5 mL) was added. The suspension was gently stirred at room temperature and the progress of the reaction was monitored by TLC. After 4 days, the resin was filtered and thoroughly washed with THF/H_2O, H_2O, and THF. All the washings were pooled and extracted with $CHCl_3$ to remove dibenzofulvene. The aqueous layer was concentrated to afford pure aminohydrazide compound **4** (60 mg, 74% yield) as a colorless oil. Compound **4** was stored under argon at 4 °C: ^1H NMR (300 MHz, D_2O) δ 1.22−1.31 (m, 15 H), 1.50 (m, 4 H), 2.15 (t, $J = 7.2$ Hz, 2 H), 2.76 (t, $J = 7.2$ Hz, 2 H); ^{13}C NMR (75 MHz, D_2O) δ 24.4, 25.0, 27.4, 33.1, 39.4, 82.9, 157.0, 176.5; *m/z* (CI/NH_3) 246 (100%, $[M + H]^+$); HRMS (CI/CH_4) *m/z* calcd for $C_{11}H_{24}O_3N_3$ $(M + H)^+$ 246.1818, found 246.1824.

Synthesis of PAMAM G4−$Fp_yNHAc_z(ArHydr\text{-}NHBoc)_{x'}$ (Conjugate 5). MeOH was evaporated from conjugate **3** solution (1.1 μmol of G4, 5.5 μmol of $ArNO_2$). CF_3CH_2OH (1 mL) was quickly added then evaporated (three times). DMF (0.8 mL) was then added, followed by DIPEA (2.3 μL, 13.2 μmol) and TSTU (1.99 mg, 6.6 μmol), and the solution was stirred at room temperature in the dark for 1.5 h. Compound **4** (2.7 mg, 11 μmol) was then added in DMF (50 μL) and the solution was stirred for 24 h. The solution was diluted with MeOH (1 mL) and dialyzed extensively in MeOH using the dialysis membrane until the dialysis solvent no longer absorbed in the 200−500 nm range (4−5 days). The solution was then evaporated as described for conjugate **1** and analyzed for characterization by ^1H NMR in MeOH-d_4 (Figure 1, spectrum D, see Results and Discussion). Furthermore, conjugate **5** was analyzed by FT-IR spectroscopy to quantify the concentration of Fp entities as described above. Conjugate **5**, termed G4−$Fp_yNHAc_z(ArHydr\text{-}NHBoc)_{x'}$, was obtained in 70% yield and stored in methanol at −20 °C.

Synthesis of PAMAM G4−$Fp_yNHAc_z(ArHydr\text{-}NH_2)_{x'}$ (Conjugate 6). MeOH was evaporated from conjugate **5** solution (0.93 μmol of G4). CF_3CH_2OH (353 μL) was quickly added, followed by H_3PO_4 (47 μL, 85% in H_2O). The reaction mixture was stirred at room temperature in the dark for 2.75 h (a precipitate formed almost instantaneously). The reaction mixture was then diluted with H_2O (350 μL) and the pH was adjusted to $\approx$7 with DIPEA (160 μL) to yield a clear orange solution. To check the removal of the N-Boc protecting group by ^1H NMR in MeOH-d_4, the following procedure was carried out: the solution was transferred into a dialysis membrane and dialyzed extensively in MeOH/H_2O (1:1) for 3−4 days and then in MeOH for 48 h. ^1H NMR in MeOH-d_4 is displayed in Figure 1 (spectrum E). Furthermore, conjugate **6** was analyzed by FT-IR spectroscopy to quantify the concentration of Fp entities as described above. Conjugate **6**, termed G4−$Fp_yNHAc_z(ArHydr\text{-}NH_2)_{x'}$, was obtained in 65% yield.

Synthesis of 4-Butylamino-3-nitrobenzoic Acid (7). DIPEA (282 μL, 1.62 mmol) and *n*-butylamine (160.2 μL, 1.62 mmol) were added to a solution of 4-fluoro-3-nitrobenzoic acid (0.15 g, 0.81 mmol) in methanol (8 mL). The solution was stirred at room temperature for 48 h. H_2O was then added (20 mL) and the solution was acidified with 6 N HCl. The precipitate was filtered off, rinsed with acidic water, dissolved in ethyl acetate, and dried (Na_2SO_4). Compound **7**, obtained as a yellow powder (0.17 g, 90%), was pure enough for its application as a model compound mimicking the end branch of the dendrimer: ^1H NMR (300 MHz, DMSO-d_6) δ 0.85 (t, $J = 7.2$ Hz, 3 H), 1.28 (m, 2 H), 1.53 (m, 2 H), 3.32 (td, $J = 6.9$ and 6.2 Hz, 2 H), 7.03 (d, $J = 9.2$ Hz, 1 H), 7.87 (dd, $J = 9.2$ and 2.0 Hz, 1 H), 8.4 (t, $J = 5.8$ Hz, NH), 8.52 (d, $J = 2.0$ Hz, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 13.6, 19.5, 30.2, 42.1, 114.5, 116.8, 128.4, 130.3, 136.0, 147.2, 165.8; *m/z* (EI) 238 (40%, $[M]^{+\bullet}$), 195 (100%, $[M - C_3H_7]^+$).

Results and Discussion

Molecular Engineering Strategy. Our new hydrazide-based trifunctional G4-PAMAM dendrimer bearing the Fp probe for FT-IR detection and quantification is depicted in Scheme 1.

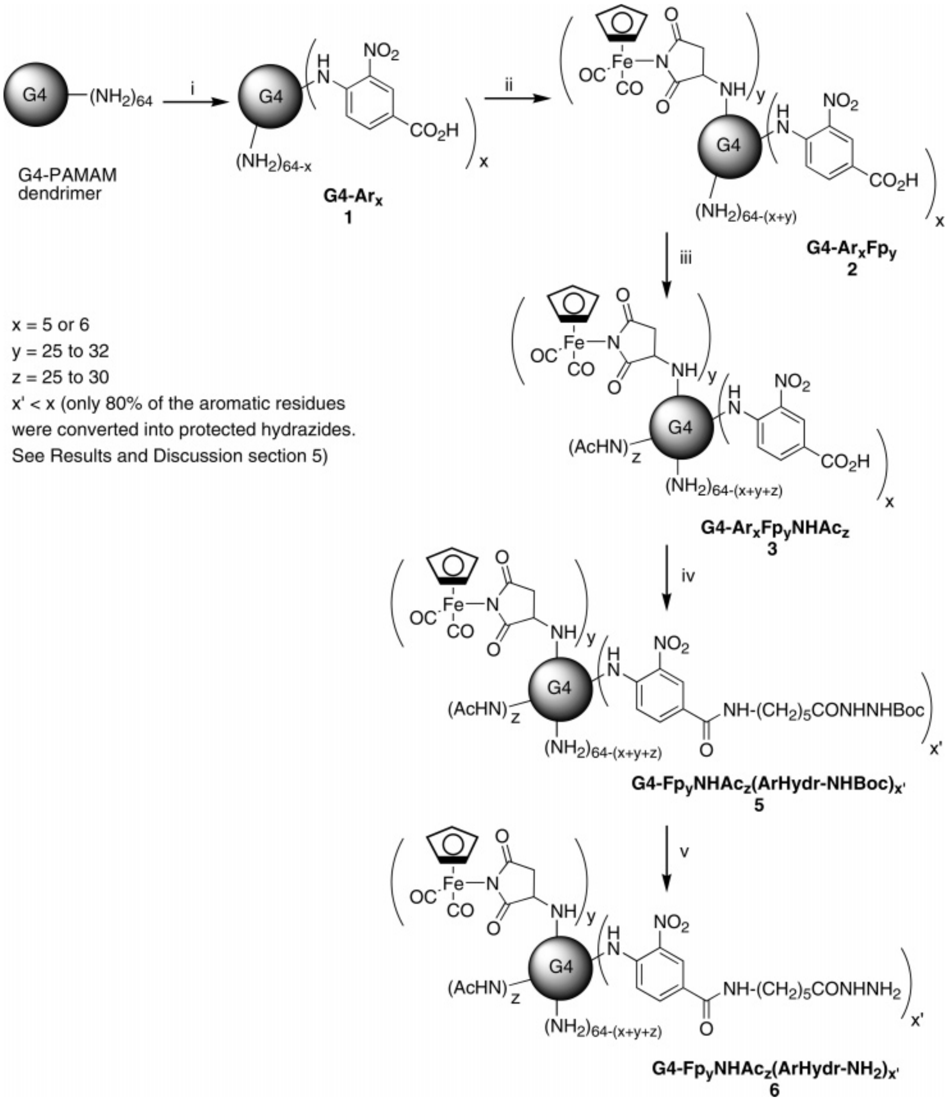
It has been designed so as to introduce an aminocaproic spacer arm between the dendrimer and the IgG-type antibody to be labeled. This spacer carries at the carboxyl end group a hydrazide function, which is known to react faster than amines with aldehydes to form stable hydrazone links. Moreover, coupling with oxidized IgG can be performed at slightly acidic pH, thus avoiding the self-condensation of oxidized IgG that could occur at neutral or basic pH when performing the coupling with amines. The amino end group of the aminocaproic acid spacer was linked to the dendrimer via a nitroaromatic carboxylic acid compound. This derivative has been shown to be useful as a UV−vis probe,[26,29,30] and we also planned to use it as an NMR probe to quantify the number of substituents conjugated. We also chose to partially acetylate the remaining surface amines. This acetylation step has three functions: (1) Blocking the remaining reactive amino groups from further reaction with electrophiles, (2) decreasing nonspecific interactions by neutralization of the dendrimer surface, and (3) increasing the water solubility of the engineered dendrimer, as well as its solubility in organic solvents such as DMSO.[31] Majoros et al. studied the stoichiometry required to achieve various degrees of acetylation of G5-PAMAM dendrimer.[32] When preparing multifunctionalized acetylated dendrimers, they started first with partial acetylation, before introducing drug, targeting agent, and imaging agent.[31,33]

The order by which the various substituents were successively introduced onto the dendrimer proved to be crucial. Conversely to Majoros' work,[31] acetylation was performed after the addition of the metal−carbonyl complex and the 4-fluoro-3-nitrobenzoic acid. Indeed, when we tried the addition of the metal−carbonyl complex first, followed by partial acetylation to yield the conjugate termed G4−$Fp_{20}NHAc_{25}$ (thus allowing about 19 amine functions for further reaction), it was not possible to further conjugate any 4-fluoro-3-nitrobenzoic acid molecule, even with 15 equiv for 4 days. As the objective was to use the newly engineered dendrimer as an IR probe for CMIA quantification, the functional dendrimer should carry as many Fp complexes as possible to afford amplification of the IR signal. Due to the bulkiness of the Fp−maleimide complex, it must be added in the first steps of the synthesis, to be sure to tether the largest number of IR probes. In this context, the 4-fluoro-3-nitrobenzoic acid substituent (to serve as NMR standard) was introduced in the first step and then the metal−carbonyl complex, ending with the acetylation of the remaining accessible amino end groups (Scheme 2).

Synthesis. 1. Preparation of G4−Ar_x (1). Addition of 4-fluoro-3-nitrobenzoic acid afforded chromophoric *o*-nitroaniline substituents by aromatic nucleophilic substitution of the fluorine by some of the surface amines of the dendrimer.[26,29,30] Due to the hydrophobic character of the nitroaromatic ring, we decided to link only five or six nitrobenzoic residues per dendrimer. The reaction was performed in methanol with DIPEA for 48 h, and then the reaction mixture was purified by dialysis in methanol to yield G4−Ar_x **1**. The coupling extent was determined by ^1H NMR spectroscopy in methanol-d_4 (Figure 1, spectrum A).

Starting with 5 mol equiv of 4-fluoro-3-nitrobenzoic acid, it was expected that five nitroaromatic molecules would be attached per dendrimer. First, the broadening of the aromatic proton peaks at 8.76, 8.07, and 7.08 ppm indicated that the nitroaromatic substituent was covalently bound to the dendrimer. Then, on the basis of the integration values of one of the three aromatic peaks normalized to 1 and the 248 methylene protons next to the carbonyl groups of the dendrimer assigned at 2.3 ppm (assuming that the dendrimer was ideally synthesized, i.e., without branch defect), five nitroaromatic molecules per den-

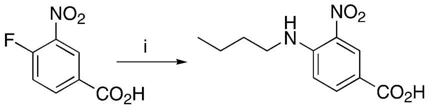
Scheme 2. Synthetic Route to the Hydrazide-Based Trifunctional G4-PAMAM Dendrimer Bearing the (η^5-Cyclopentadienyl) Iron Dicarbonyl FT-IR Probe[a]



[a] (i) 4-Fluoro-3-nitrobenzoic acid, DIPEA, MeOH; (ii) Fp-maleimide, MeOH; (iii) Ac_2O, CF_3CH_2OH; (iv) TSTU, DIPEA, DMF then $H_2N(CH_2)_5CONHNHBoc$ **4**; (v) H_3PO_4, CF_3CH_2OH.

drimer was calculated, i.e., equal to what was expected. Starting with 6 mol equiv of 4-fluoro-3-nitrobenzoic acid, a coupling ratio of 6 was found (data not shown). These results show that this conjugation step can be precisely tuned by modulating the molar ratio of the reactants. All the synthesized dendrimers must be kept in solution, here methanol, to maintain their stability, since we and others[34] have experienced their degradation when they were stored without solvent. Therefore, the concentration of the labeled dendrimers in a given solution has to be determined before using them in the next step of the synthesis. It has also been noticed that the determination of the dendrimer concentration by measuring the concentration of the remaining free amino groups on the dendrimer surface was not satisfactory, due to the shielding of some free amines[26,35] and possible defects during the synthesis of G4-PAMAM dendrimer that lead to fewer arms per generation.[3,32] Consequently, the concentration of the modified dendrimer was indirectly determined by measuring the concentration of the conjugated nitroaniline chromophore by UV−vis spectroscopy at 428 nm using a calibration curve constructed with 4-butylamino-3-nitrobenzoic acid **7** (Scheme 3) that mimics the nitroaromatic derivative attached to the dendrimer. Then, the concentration of dendrimer in each solution was easily deduced from the nitrobenzoic acid to G4 ratio determined by NMR. The yield of this first step was around 80%. This result shows that dialysis is a more efficient purification technique, affording higher recoveries than the previously used lipophilic gel-permeation chromatography (GPC) on Sephadex LH 20 that led to only 50% recovery.[26,27]

Scheme 3. Model Compound 7 Mimicking the Dendrimer End Branches Grafted with the UV−Vis/NMR Probe[a]



7

[a] (i) $nBuNH_2$, DIPEA, MeOH.

2. Preparation of G4−Ar_xFp_y (2). The second step was the conjugation of the Fp−maleimide complex[28] by Michael addition of some of the surface amines of the dendrimer to the maleimide double bond as previously described.[25,27] The reaction mixture was purified by dialysis in methanol instead of GPC on Sephadex LH 20,[27] with several changes of methanol until all unbound Fp−maleimide passed through the dialysis mem-

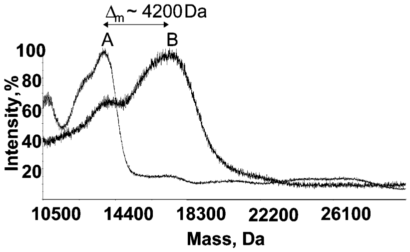


Figure 2. MALDI-TOF mass spectra of G4-PAMAM (A) and G4−Ar_6Fp_{32} (B).

brane. This purification step was monitored by UV−vis analysis of the dialysis solvent in the 200−500 nm range. Four to 5 days were necessary to fully eliminate excess Fp−maleimide. The coupling extent was again determined by NMR spectroscopy in methanol-d_4 (Figure 1, spectrum B). The peak at 5.2 ppm was clearly assigned to the five protons of the cyclopentadienyl entity of the metal−carbonyl unit. The comparison of the intensity of this peak, related to one proton, with one of the aromatic peaks led to an average extent of conjugation of 25−32 Fp complexes per dendrimer, starting with 40 molar equiv of Fp−maleimide. To check the coupling extent determined by NMR measurements, a MALDI-TOF mass spectrometry experiment was carried out with a more loaded G4−Ar_6Fp_{32} conjugate.

Figure 2 shows the superimposition of the mass spectra of commercial G4-PAMAM (spectrum A) and of G4−Ar_6Fp_{32} dendrimer (spectrum B). PAMAM dendrimers higher than generation 3 display broad signals in MALDI-TOF MS experiments, as shown in previous reports.[36,37] The expected mass difference is $m/z = 9726$, whereas the observed difference is $m/z = 4200$. Taking into account the UV sensitivity of $CpFe(CO)_2$−succinimidyl derivatives, we can assume that the weak Fe−N (from succinimidyl) bond is cleaved under laser irradiation during the ionization process, as the susceptibility of such metal−carbonyl derivatives toward mass spectrometry analysis has been previously noticed by our group.[38] Thus, by postulating that $CpFe(CO)_2$ entities were lost during the process, only 32 succinimidyl moieties were still tethered onto the dendrimer surface as well as six nitro aromatic derivatives. The new calculated mass difference becomes $(32 \times 98) + (6 \times 165) = 4126$. This value is now consistent with the observed average mass shift. The MALDI-TOF mass spectrometry experiment thus confirms the results estimated by ^1H NMR spectroscopy.

This time, the concentration of the dendrimer in methanolic solution could not be determined by measuring the concentration of the nitroaniline chromophore by UV−vis spectroscopy at 428 nm, as we observed an overlapping with the absorption of the Fp complex ($\lambda_{max} = 365$ nm). Therefore, we decided to quantify the Fp entity by FT-IR spectroscopy. Taking advantage of the specificity of the transition-metal−carbonyl complexes that display intense absorption bands in the mid-infrared spectral range (1800−2200 cm^{-1}), owing to the stretching modes νCO of the carbonyl ligands coordinated to the metal, we had previously shown that it was possible to quantify such complexes either in solution[21,39] or deposited on nitrocellulose[27] or nylon membranes[26] by measuring the νCO peaks heights that were proportional to the quantity of the complex. This is in fact the basis of the CMIA method.[23] In the 1800−2200 cm^{-1} range, the IR spectrum of Fp−maleimide displays two intense bands at 2052 and 2000 cm^{-1} that are characteristic of the symmetric and asymmetric stretching modes of the carbonyl ligands (Figure 3a, spectrum A). A calibration curve was constructed by plotting the absorbance of the 2052 cm^{-1} peak termed the "analytical peak", as it was shown to be the most sensitive,[39] versus standard concentrations of the Fp−maleimide in buffered sodium phosphate solution spotted onto nitrocellulose membranes. Figure 3b shows the typical calibration straight line of Fp−maleimide in the range 0.05−0.66 μmol/mL in 10 mM phosphate buffer pH 7.2 that allowed the quantification of Fp entities in the dendrimer solution (Figure 3a, spectrum C). Then, from the number of Fp entities bound to the dendrimer calculated from ^1H NMR measurements, the concentration of conjugate **2** and the yield of the reaction (80%) were easily deduced.

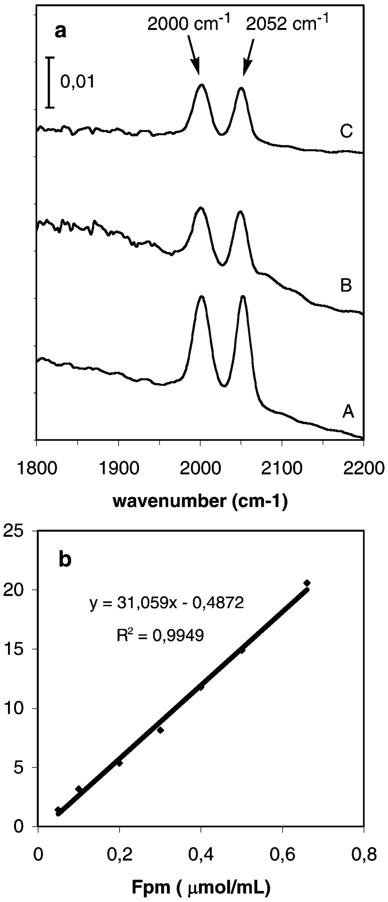
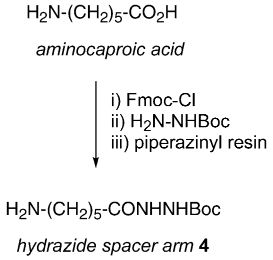


Figure 3. (a) FT-IR spectra on nitrocellulose membranes. Four microliters of the following solutions were deposited and left to dry. Spectrum A: 0.66 μmol/mL standard solution of $CpFe(CO)_2$-maleimide, corresponding to the upper limit of the calibration curve shown in (b). Spectrum B: fully functionalized dendrimer **6** after treatment with H_3PO_4. Spectrum C: bifunctionalized $G4Ar_5Fp_{26}$.

3. Preparation of G4−$Ar_xFp_y(NHAc)_z$ (3). Acetylation of the remaining free amino groups on the dendrimer surface was then performed with an excess of acetic anhydride and DIPEA in trifluoroethanol. This non-nucleophilic alcohol was preferred to methanol to avoid the formation of methyl ester from reaction of methanol with the mixed anhydride that could be formed from nitrobenzoic acid derivative and acetic anhydride. Purification was done by extensive dialysis in methanol/water to remove the alkylammonium acetate salt and then in methanol to yield conjugate **3**, termed G4−$Ar_xFp_yNHAc_z$. Characterization of this conjugate was again performed by combining ^1H NMR and FT-IR spectroscopies. The ^1H NMR spectrum of the conjugate in methanol-d_4 (Figure 1, spectrum C) shows first the peak at 1.95 ppm related to the CH_3 protons of the acetyl group. Second, the integral ratio between the signal of the Cp at 5.2 ppm and the aromatic proton at 8.76 ppm normalized to 1 was unchanged compared to the ratio before acetylation, indicating that the reaction conditions did not affect the stability of the Fp complex.

Scheme 4. Synthetic Route to the Hydrazide Spacer Arm 4



The average number of acetyl residues attached per dendrimer was calculated from the integral ratios, knowing that integration of one aromatic proton normalized to one is related to five aromatic residues per dendrimer. Between 25 and 30 acetyl residues were attached per dendrimer, which meant that almost all the surface amines were modified, since an average number of 25−32 Fp units per dendrimer was found at the previous step. Then, FT-IR quantification of the Fp entity was performed as described for conjugate **2** to calculate the concentration of G4-PAMAM in solution and the reaction yield (75%).

4. Synthesis of the Aminohydrazide Spacer Arm 4. The aminohydrazide spacer arm **4** was prepared as described in Scheme 4. Aminocaproic acid was first treated with FmocCl to afford the N-protected amino acid in 91% yield, and then the carboxylic acid end group was activated with TSTU and allowed to react with *tert*-butyl carbazate to afford the *N*-Boc protected hydrazide derivative in 72% yield. Cleavage of the Fmoc protecting group was cleanly achieved by incubation with a piperazinyl resin[40,41] in THF/H_2O (3:1)[42] to afford the expected aminohydrazide compound **4** in 74% yield. This solid-phase procedure was favored as the high water solubility of the aminohydrazide **4** precluded clean purification from excess of amine base used in classical cleavage technique.[43]

5. Preparation of G4−$Fp_y$$NHAc_z$$(ArHydr\text{-}NHBoc)_{x'}$ (5). Addition of the aminohydrazide spacer arm was performed in DMF, after activation of the carboxylic functions of the nitrobenzoic residues with TSTU. The reaction conditions and the reaction time were optimized using the model compound 4-butylamino-3-nitrobenzoic acid **7** (Scheme 3), with the *n*-BuNH substituent mimicking the end branch of the dendrimer. Again, the reaction mixture was extensively dialyzed in methanol with a daily methanol change to remove DMF and all the organic compounds. The fully functionalized dendrimer **5** was again characterized by combining ^{1}H NMR and FT-IR spectroscopies. The ^{1}H NMR spectrum in methanol-d_4 (Figure 1, spectrum D) shows first the peak at 1.45 ppm related to the *t*-Bu protons of the protected hydrazide. In the aromatic area, two signals are visible for each proton. Signals at 8.76, 8.10, and 7.08 ppm were attributed to the starting nitrobenzoic acid residue not converted into amide, whereas signals at 8.7, 8.0 and 7.17 ppm were attributed to the expected amidohydrazide spacer arm. The variation of the chemical shifts observed between acid and amide species was identical to that observed when performing the reaction with the model compound (data not shown). Around four out of five of the aromatic residues were estimated to have been converted into protected hydrazide derivatives. The integral ratio between the Cp signal at 5.2 ppm and the aromatic (acid + amide) proton was still unchanged, indicating that there was no loss of metal−carbonyl complex during amide bond formation. The concentration of dendrimer and the reaction yield (70%) were again calculated by FT-IR spectroscopy, from the absorbance at 2052 cm^{-1}. It is noteworthy that, at this step, these conjugates are fully soluble in methanol and methanol-d_4 as opposed to previous samples prepared without the acetylation step (data not shown) that were poorly soluble in methanol and precipitated in methanol-d_4. Acetylation of the remaining amino groups indeed resulted in increased solubility of our trifunctional dendrimers in organic solvents, as reported by others.[31,33]

6. Deprotection of the *N*-Boc-Protected Hydrazide Dendrimer 5. Numerous methods have been reported for the cleavage of the *N*-Boc group, including the use of strong acids such as CF_3CO_2H, HCl, or H_2SO_4.[43] We studied first the stability of the G4−Ar_xFp_y dendrimer **2** in these commonly used conditions. The labeled dendrimer was partly insoluble after workup, and FT-IR spectroscopy of the crude solution showed a large decrease of the absorbance of the νCO bands of the Fp label compared to the initial compound, indicating a substantial loss of metal−carbonyl complex and/or a very low recovery of dendrimer. Fortunately, phosphoric acid, a weaker acid ($pK_{a1} = 2.15$) than trifluoroacetic acid ($pK_a = 0.3$), has been recently reported to remove the *N*-Boc group in mild conditions.[44] This time, the Fp-labeled dendrimer was fully soluble after neutralization of the reaction mixture, and the FT-IR analysis of the solution after dialysis showed clean νCO bands (Figure 3a, spectrum B). The absorbance measured at 2052 cm^{-1}, before and after H_3PO_4 treatment, indicated a good stability of the Fp moieties submitted to these acidic conditions, although a slight decrease of the absorbance was observed. This preliminary study being done, the precise reaction conditions for the removal of the *N*-Boc group were studied with the model compound **7**, having the hydrazide spacer arm, since the outcome of the reaction is highly dependent on the concentration of the reactants.[44] Typically, the protected dendrimer was allowed to react with aqueous 85 wt % phosphoric acid diluted 8.5 times in trifluoroethanol for 2.75 h. After addition of water and neutralization with DIPEA, the crude solution was dialyzed extensively in methanol/water (1:1) and then in methanol to check the removal of the *N*-Boc groups by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum in methanol-d_4 (Figure 1, spectrum E) shows first the disappearance of the peak at 1.44 ppm, indicating that the cleavage of the Boc group was complete within the conditions stated for the model compound. The integral ratio between the Cp signal and one aromatic proton signal is slightly lower, confirming the preliminary results observed by FT-IR spectroscopy. This loss of metal−carbonyl complexes was estimated to be around 8%. In the aromatic protons peaks area, the acid/amide ratio changed unexpectedly after H_3PO_4 treatment. We could exclude hydrolysis of the amide linkage, since no such reaction was observed with the model compound. We hypothesize that the fraction of dendrimer molecules carrying the largest proportion of free hydrazide-terminated spacer arms is less soluble in methanol-d_4 or tend to stick on the dialysis membrane, which remained yellow even after thorough rinsing. The reaction yield (65%), again calculated by FT-IR spectroscopy, confirmed that a lower recovery was obtained after this cleavage step. Nevertheless, there are still enough free hydrazide groups at the end of the spacer arm for further conjugation. Furthermore, with the reaction conditions now being established, a different procedure will be carried out when performing the coupling with aldehyde-containing molecules. After reaction with H_3PO_4 and neutralization, dendrimer carrying free hydrazide will be directly allowed to react with its coupling counterpart without dialysis and NMR checking.

Conclusion

We have shown that PAMAM dendrimers are valuable tools for the design of new metal−carbonyl multilabeled reagents intended for conjugation to immunological material and as infrared probes for the amplification of the IR signal in carbonyl metalloimmunoassay. This new synthesis has taught us several things: (1) conjugation of the nitroaniline derivative to introduce carboxylic acid functions proved to be a breakthrough in the characterization of the labeled dendrimers, making the determination of coupling rates by ^{1}H NMR spectroscopy easier; (2) The MALDI-TOF MS experiment confirmed the average coupling rate estimated by ^{1}H NMR spectroscopy, showing that NMR spectroscopy is a reliable technique for the chemical characterization of such polymers, provided that distinct chemical shifts are available for the molecules linked to the dendrimer's peripheral amine functions; (3) dialysis also proved to be a good purification technique for such compounds, since good yields were obtained with clean ^{1}H NMR spectra. These multilabeled dendrimers prove to be as soluble in aqueous buffer as in organic media such as methanol or DMF. They are loaded with a large number of Fp units for amplification of the IR signal and a hydrazide-terminated spacer arm for further optimized conjugation to oxidized secondary antibodies. These Fp-labeled secondary antibodies will be studied as universal detection reagents for the development of a new solid-phase format for the carbonyl metalloimmunoassay.

Acknowledgment. The Centre National de la Recherche Scientifique and the French Ministry of Research are gratefully acknowledged for financial support.

References and Notes

(1) Tomalia, D. A.; Baker, H.; Dewald, J. R.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Polym. J.* **1985**, *17*, 117−132.
(2) Tomalia, D. A.; Baker, H.; Dewald, J. R.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Macromolecules* **1986**, *19*, 2466−2468.
(3) Tomalia, D. A.; M., N. A.; Goddard, W. A., III *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138−175.
(4) Svenson, S.; Tomalia, D. A. *Adv. Drug Delivery Rev.* **2005**, *57*, 2106−2129.
(5) Gillies, E. R.; Fréchet, J. M. J. *Drug Discovery Today* **2005**, *10*, 35−43.
(6) Venditto, V. J.; Regino, C. A. S.; Brechbiel, M. W. *Mol. Pharm.* **2005**, *2*, 302−311.
(7) Boas, U.; Heegaard, P. M. H. *Chem. Soc Rev.* **2004**, *33*, 43−63.
(8) Stiriba, S.-E.; Frey, H.; Haag, R. *Angew. Chem., Int. Ed.* **2002**, *41*, 1329−1334.
(9) Esfand, R.; Tomalia, D. A. *Drug Discovery Today* **2001**, *6*, 427−436.
(10) Patri, A. K.; Majoros, I. J.; Baker, J. R., Jr. *Curr. Opin. Chem. Biol.* **2002**, *6*, 466−471.
(11) Paleos, C. M.; Tsiourvas, D.; Sideratou, Z. *Mol. Pharm.* **2007**, *4*, 169−188.
(12) Won, B. Y.; Choi, H. G.; Kim, K. H.; Byun, S. Y.; Kim, H.-S.; Yoon, H. C. *Biotechnol. Bioeng.* **2005**, *89*, 815−821.
(13) Hong, M.-Y.; Lee, D.; Kim, H.-S. *Anal. Chem.* **2005**, *77*, 7326−7334.
(14) Hong, M.-Y.; Yoon, H. C.; Kim, H.-S. *Langmuir* **2003**, *19*, 416−421.
(15) Snejdarkova, M.; Svobodova, L.; Evtugyn, G.; Budnikov, H.; Karyakin, A.; Nikolelis, D. P.; Hianik, T. *Anal. Chim. Acta* **2004**, *514*, 79−88.
(16) Snejdarkova, M.; Svobodova, L.; Nikolelis, D. P.; Wang, J.; Hianik, T. *Electroanalysis* **2003**, *15*, 1185−1191.
(17) Svobodova, L.; Snejdarkova, M.; Hianik, T. *Anal. Bioanal. Chem.* **2002**, *373*, 735−741.
(18) Grabchev, I.; Chovelon, J.-M.; Nedelcheva, A. *J. Photochem. Photobiol. A* **2006**, *183*, 9−14.
(19) Sali, S.; Grabchev, I.; Chovelon, J.-M.; Ivanova, G. *Spectrochim. Acta A.* **2006**, *65*, 591−597.
(20) Chen, Q. Q.; Lin, L.; Chen, H. M.; Yang, S. P.; Yang, L. Z.; Yu, X. B. *J. Photochem. Photobiol. A* **2006**, *180*, 69−74.
(21) Salmain, M.; Vessières, A.; Jaouen, G.; Butler, I. S. *Anal. Chem.* **1991**, *63*, 2323−2329.
(22) Salmain, M.; Vessières, A.; Brossier, P.; Butler, I. S.; Jaouen, G. *J. Immunol. Methods* **1992**, *148*, 65−75.
(23) Vessières, A.; Salmain, M.; Brossier, P.; Jaouen, G. *J. Pharm. Biomed. Anal.* **1999**, *21*, 625−633.
(24) Salmain, M.; Vessières, A. In *Bioorganometallics, biomolecules, labelling, medicine;* Jaouen, G., Ed.; Wiley-VCH: Weinheim, Germany, 2006; pp 263−302.
(25) Salmain, M.; Fischer-Durand, N.; Cavalier, L.; Rudolf, B.; Zakrzewski, J.; Jaouen, G. *Bioconjugate Chem.* **2002**, *13*, 693−698.
(26) Heldt, J.-M.; Fischer-Durand, N.; Salmain, M.; Vessières, A.; Jaouen, G. *J. Organomet. Chem.* **2004**, *689*, 4775−4782.
(27) Fischer-Durand, N.; Salmain, M.; Rudolf, B.; Vessières, A.; Zakrzewski, J.; Jaouen, G. *ChemBioChem* **2004**, *5*, 519−525.
(28) Rudolf, B.; Zakrzewski, J. *Tetrahedron Lett.* **1994**, *35*, 9611.
(29) Ladd, D. L.; Snow, R. A. *Anal. Biochem.* **1993**, *210*, 258−261.
(30) Shukla, S.; Wu, G.; Chatterjee, M.; Yang, W.; Sekido, M.; Diop, L. A.; Müller, R.; Sudimack, J. J.; Lee, R. J.; Barth, R. F.; Tjarks, W. *Bioconjugate Chem.* **2003**, *14*, 158−167.
(31) Majoros, I. J.; Thomas, T. P.; Mehta, C. B.; Baker, J. R., Jr. *J. Med. Chem.* **2005**, *48*, 5892−5899.
(32) Majoros, I. J.; Keszler, B.; Woehler, S.; Bull, T.; Baker, J. R., Jr. *Macromolecules* **2003**, *36*, 5526−5529.
(33) Majoros, I. J.; Myc, A.; Thomas, T. P.; Mehta, C. B.; Baker, J. R., Jr. *Biomacromolecules* **2006**, *7*, 572−579.
(34) Baker, W. S.; Lemon, B. I., III; Crooks, R. M. *J. Phys. Chem. B.* **2001**, *105*, 8885−8894.
(35) Luo, D.; Haverstick, K.; Belcheva, N.; Han, E.; Saltzman, W. M. *Macromolecules* **2002**, *35*, 3456−3462.
(36) Shi, X.; Lesniak, W.; Islam, M. T.; C., M. M.; Balogh, L. P.; Baker, J. R., Jr. *Colloids Surf. A* **2006**, *272*, 139−150.
(37) Hong, M.-Y.; Kim, Y.-J.; Lee, J. W.; Kim, K.; Lee, J.-H.; Yoo, J.-S.; Bae, S.-H.; Choi, B.-S.; Kim, H.-S. *J. Colloid Interface Sci.* **2004**, *274*, 41−48.
(38) Haquette, P.; Salmain, M.; Svedlung, K.; Martel, A.; Rudolf, B.; Zakrzewski, J.; Cordier, S.; Roisnel, T.; Fosse, C.; Jaouen, G. *ChemBioChem* **2007**, *8*, 224−231.
(39) Vessières, A.; Kowalski, K.; Zakrzewski, J.; Stepien, A.; Grabowski, M.; Jaouen, G. *Bioconjugate Chem.* **1999**, *10*, 379−385.
(40) Carpino, L. A.; Mansour, E. M. E.; Knapczyk, J. *J. Org. Chem.* **1983**, *48*, 666−669.
(41) Carpino, L. A.; Williams, J. R.; Lopusinski, A. *J. Chem. Soc., Chem. Commun.* 1978, 450.
(42) Santini, R.; Griffith, M. C.; Qi, M. *Terahedron Lett.* **1998**, *39*, 8951−8954.
(43) Greene, T. W.; Wuts, P. G. M. *Protective groups in organic synthesis*; John Wiley and Sons: New York, 1999.
(44) Li, B.; Bemish, R.; Buzon, R. A.; Chiu, C. K.-F.; Colgan, S. T.; Kissel, W.; Le, T.; Leeman, K. R.; Newell, L.; Roth, J. *Tetrahedron Lett.* **2003**, *44*, 8113−8115.

MA071621G