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[9] Compound **5** (150 mg, 6.1×10^{-5} mol) and compound **A** (1.3 g, 1.64×10^{-3} mol) were dissolved in dry toluene (10 mL). Fifteen drops of a solution of Karstedt's catalyst in xylenes (3.5%; Fluorochem) were added and the reaction mixture was stirred overnight at 45 °C. Volatiles were evaporated and the residue was purified by column chromatography on alumina (Brockmann II). The polarity of the eluent was varied from hexane/dichloromethane ratios of 3/1 to 1/3. Yield of isolated product: 60 mg (8%). ^{1}H NMR (399.7 MHz, CD_2Cl_2, 25 °C): $\delta = 0.01$ (s, 144H; -OSi(CH_3)$_2$-), 0.16–0.17 (5s (not resolved), 180H; NSi(CH_3)$_2$-), 0.35–0.60 (m, 108H; SiCH_2-), 0.88 (2t (not resolved), 72H; -CH_3), 1.2–1.6 (m, -CH_2-, 360H), 1.75–1.84 (m, 72H; CH_2CH$_2$O), 4.00 (3t (not resolved), -CH_2O), 6.51 (m, 24H; CH, (carbonyl)), 6.95, 7.20, 7.50, 7.56 (4d, 4×24H; CH, biphenyl unit), 7.98 (d, 12H; CH, (carbonyl)); ^{13}C NMR (100.4 MHz, CD_2Cl_2, 25 °C): $\delta = -0.18$, 0.53 (-Si(CH_3)$_2$O-Si(CH_3)$_2$-), 3.43, 3.50 (-N{Si(CH_3)$_2$-}$_2$), 11.1, 12.7 (NSi(Me)$_2$CH_2CH_2-Si(Me)$_2$O-), 14.0 (NSi(Me)$_2$CH_2CH_2Si(Me)$_2$N-), 14.3 (CH_3), 18.7 (CH_2Si(Me)$_2$O), 23.1–32.3 (-CH_2-), 68.5, 68.7, 69.3 (-CH_2O), 100.5, 105.5, 111.6, 115.1, 122.6, 127.7, 128.3, 133.0, 134.5, 138.5, 150.6, 159.2, 161.9, 164.8 (C aromatic), 164.3 (-*C*(O)O); GPC (THF, toluene standard) ($\langle M_w \rangle / \langle M_n \rangle$) = 1.024.

Spin-Labeled Dendrimers in EPR Imaging with Low Molecular Weight Nitroxides

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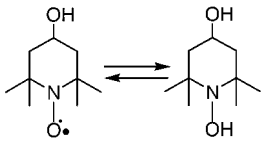
Electron paramagnetic resonance (EPR) imaging is an emerging technology that shows great promise in medical research applications for measuring free radical distribution, metabolism, and extent of oxygenation in tumors, organs and tissues.[1] However, the stable nitroxyl radicals (nitroxides) which these applications utilize, such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), are prone to bioreduction to the analogous diamagnetic, EPR-silent hydroxylamines (i.e., TEMPOL-H; Scheme 1). The nitroxide and the hydroxylamine species rapidly establish an equilibrium that strongly favors the hydroxylamine in vivo.[2] Thus, the short half-life of nitroxides in vivo ($t_{1/2} \approx 3$ min) has limited further development and wide application of EPR imaging in the biomedical field.



TEMPOL TEMPOL-H

Scheme 1. The equilibrium established between TEMPOL and TEMPOL-H. The latter is favored in vivo.

Recently it was reported that intravenous injection of polynitroxyl-albumin (PNA) caused reoxidation of free hydroxylamine (shown to remain in the body for a relatively long period of time) back to the paramagnetic nitroxide.[3] This reagent has enabled high-resolution EPR imaging of rat heart and may also permit various therapeutic applications of nitroxides. Alternatively, polyamidoamine (PAMAM) Starburst™ dendrimers are spherical macromolecules which can be produced in successive generations, each with a specified and defined size and molecular weight as well as a high number of terminal primary amino groups.[4] Because of these characteristics and the low immunogenicity, PAMAMs are finding utility in a variety of applications, many of which are biomedical.[5] Due to their macromolecular nature, dendrimers are impermeable and when injected intravenously distribute primarily in the vascular space. This is in contrast to TEMPOL and TEMPOL-H, which are membrane permeable and move readily between the extra- and intracellular spaces, the latter being the major sites of nitroxide reduction.[6] Recently dendrimers labeled with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were used to evaluate the heterogenicity of their carbohydrate-substituted surfaces.[7a] Previous EPR studies of lower generation dendrimers with 2,2,5,5-tetramethylpyrrolidin-1-oxyl (PROXYL) conjugates have shown that the large spin–spin interaction encountered by dendrimer-labeled nitroxides causes extensive broadening of the EPR spectra, effectively making the latter indistinguishable from the base line.[7b] In contrast, the low molecular weight nitroxides exhibit sharp EPR spectra, and the intensities of the bands can be easily measured. Here we report the preparation and characterization of two novel spin-labeled dendrimers, a polynitroxyl G-6 PAMAM™ dendrimer with 198 (G-6-TEMPO-198) or with 80 (G-6-TEMPO-80) free TEMPO radicals on the surface of the macromolecule. We show that both dendrimers reoxidize TEMPOL-H back to its EPR-active form, TEMPOL, and therefore are potential TEMPO free radical, life-supporting agents for EPR imaging.

The commercially available G-6 PAMAM™ dendrimers are synthetic oblate spheroidal macromolecules composed of an ethylenediamine initiator core and repeating polyamidoamino units resulting in 256 amines on the surface. To attach TEMPO free radicals to the parent G-6 dendrimer, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**1**, 4-amino-TEMPO) was first converted into 2,2,6,6-tetramethylpiperidine-1-oxyl-4-succinamic acid (**2**), which subsequently reacted to form the *N*-hydroxysuccinimidyl ester **3** (Scheme 2). Compounds **2** and **3** were characterized by their exact fast atom bombardment (FAB) mass spectra. No satisfactory elemental analyses could

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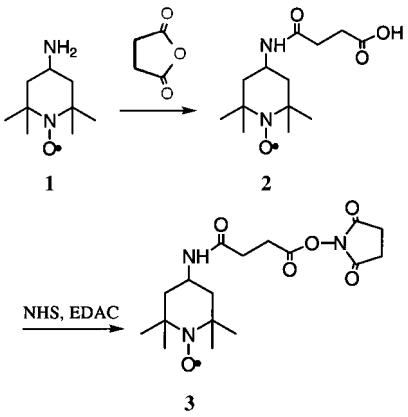


Scheme 2. Synthesis of *N*-hydroxysuccinimidyl-TEMPO active ester **3**. EDAC = 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, NHS = *N*-hydroxysuccinimide.

be obtained for these compounds due to their extremely hygroscopic nature.

Treatment of the parent G-6 PAMAM™ dendrimer with either an excess or a stoichiometric amount of **3** resulted in G-6-TEMPO-198 and G-6-TEMPO-80, in which either 198 or 80 TEMPO radicals are attached to the dendrimer (Scheme 3). The degree of substitution was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy[8] (*m*/*z*: 102 000 and 72 000, compared to 50 900 for the nonlabeled G-6 dendrimer). When mixed with TEMPOL-H, both dendrimer nitroxyl conjugates reoxidized the hydroxylamine back to TEMPOL, the EPR-active analogue, as clearly seen from the sharp increase in the intensity of the TEMPOL-produced EPR signal within minutes (Figure 1). There was no reoxidation when TEMPOL-H was mixed with the G-6 dendrimer lacking conjugation to TEMPO. Increasing the degree of labeling of the dendrimer with **3** resulted in increasing the reoxidation rate, with equilibrium being reached after 30 min with G-6-TEMPO-80 and after 20 min with G-6-198.

Since aggregation can often create particles with undesirable characteristics for in vivo applications, due to both size and high immunogenicity, a multi-angle light scattering (MALS) analysis of the two spin-labeled dendrimers was also performed. This analysis revealed that in aqueous solution the highly substituted G-6-TEMPO-198 aggregates to form particles with an average molecular weight of 549 000 (degree of aggregation ≈ 5.4) and a radius of 18.8 nm. This high degree of aggregation is attributed to the highly lipophilic nature of the G-6-TEMPO-198 surface. An aggregate of this size would most likely obstruct narrow blood capillaries in some tissues, cause precipitable masses in vivo, and thus be a source of toxicity. The MALS analysis of the less highly substituted G-6-TEMPO-80, on the other hand, revealed particles with an average molecular weight of 113 100 (degree of aggregation ≈ 1.6) and a radius of less than 10 nm. Thus, the lower degree of substitution in G-6-TEMPO-80 decreases the lipophilic nature of the surface and thus the potential for aggregation in aqueous media.

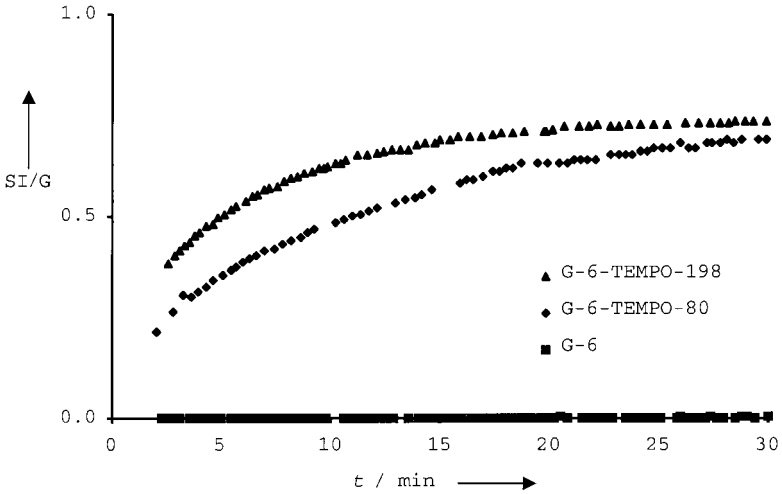
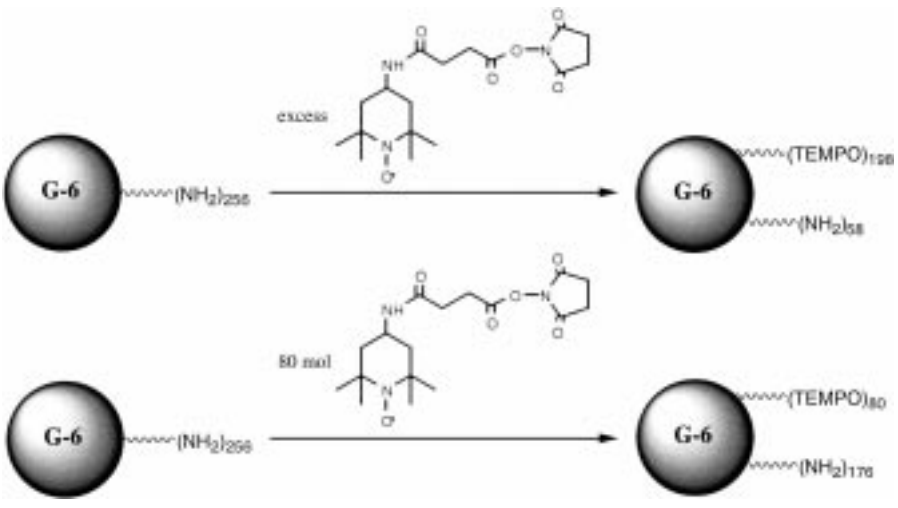


Figure 1. Plot of the intensity of the EPR signal of TEMPOL, formed by reoxidation of TEMPOL-H with spin-labeled PAMAM™ dendrimers, as a function of time *t* after addition of the dendrimer. SI = signal intensity, G = gain.



Scheme 3. Synthesis of G-6-TEMPO-198 and G-6-TEMPO-80.

Our study has shown that spin-labeled G-6 dendrimers are potential candidates for TEMPO free radical life supporters in EPR imaging. Currently, murine model studies are under way to determine the prolonging effect of G-6-TEMPO-80 on the half-life of TEMPOL in vivo and possible applications of this dendrimer in EPR imaging technics.

Experimental Section

General: All reactions were carried out under argon. Anhydrous THF, 4-amino-TEMPO, succinic anhydride, EDAC, and NHS were purchased from Aldrich Chemical Co., Milwaukee, WI. The PAMAM™ dendrimer, generation 6, ethylenediamine core, was generously provided by Dendritech Inc.,

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Midland, MI. DMSO was dried over 4-Å molecular sieves. Exact FAB mass spectra were obtained on an Extrel 4000 instrument in the positive-ion detection mode. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. The MALDI mass spectra were acquired using a PerSeptive Biosystems Voyager RP time-of-flight mass spectrometer. Positive-ion mass spectra were acquired in the linear mode, and the ions were generated by using a nitrogen laser (337 nm) pulsed at 3 Hz with a pulse width of 3 ns. Ions were accelerated at 30000 V and amplified using a multichannel plate. Spectra (70 to 180) were summed into a 500-MHz Techtronix digital storage oscilloscope and downloaded to a computer for data processing. All data processing was performed using GRAMS (Gallactic Industries, Salem, NH). Spectra of TEMPO-labeled dendrimers were obtained using a *trans*-3-indoleacrylic acid matrix with a matrix:-analyte ratio of 8000:1. Bovine serum albumin (BSA, MW = 66431 g mol^{-1}) was used as an external standard. An aliquot corresponding to 12 pmol of the analyte was deposited on the laser target.[9] The MALS analyses were performed by Wyatt Technology Corporation, Santa Barbara, CA, using a DAWN EOS detector with a solid-state laser operating at 690 nm. Nanopure water (G-6-TEMPO-80) or 17 mM acetic acid (G-6-TEMPO-198) were used as solvents. The EPR spectra were recorded on a Varian E9 X-band spectrometer with a field of 3368 G, a modulation frequency of 100 kHz, a modulation amplitude of 1 G, and a microwave power of 10 mW.

2: 4-Amino-TEMPO (**1**) was first purified by gradient elution flash chromatography ($CH_2Cl_2 \rightarrow CH_2Cl_2$/MeOH 4/1). Succinic anhydride (1.6 g) was added to the purified 4-amino-TEMPO (2.8 g) in THF (150 mL), and the reaction mixture was stirred at ambient temperature for 18 h. The solvent was evaporated to near dryness, and the residue subjected to gradient elution flash chromatography ($CH_2Cl_2 \rightarrow CH_2Cl_2$/MeOH 9/1). The hygroscopic solid was immediately used in the next step. Yield 2.1 g (47%). Exact FAB mass spectrum: 273.1797 [M^+], calcd: 273.1814.

3: Compound **2** (8.0 g) was dissolved in THF (300 mL), after which first NHS (3.45 g) and then EDAC (5.8 g) were added. The mixture was stirred at ambient temperature for 18 h. The solvent was evaporated, the residue redissolved in $CHCl_3$ (300 mL), and the mixture extracted with water (2 × 100 mL). The organic phase was then separated and dried over $MgSO_4$, and its volume reduced to about 10 mL. The product was isolated by gradient elution flash chromatography ($CH_2Cl_2 \rightarrow CH_2Cl_2$/MeOH 9/1). Yield 8.5 g (78%). Exact FAB mass spectrum: 370.1972 [M^+], calcd: 370.1978.

General procedure for the preparation of G-6-TEMPO-198 and G-6-TEMPO-80: A 10% stock solution of G-6 PAMAM™ dendrimer (15 mL) was lyophilized and the resultant solid heated to 80 °C in DMSO (200 mL) to affect complete dissolution. A solution of active ester **3** (6.0 g or 0.98 g, respectively) in DMSO (100 mL) was then added, followed by triethylamine (100 mL). The reaction mixture was then stirred at 90 °C for 72 h. After cooling, the triethylamine was removed by evaporation and the solution diluted with 10% aqueous acetic acid (300 mL) and methanol (200 mL). Unchanged active ester and *N*-hydroxysuccinimide were removed by ultrafiltration with deionized water by using a stirred cell (Amicon, MA) fitted with a 30-K membrane (Filtron, MA). Occasional precipitation of a solid material in the cell occurred. In such cases, the precipitate was brought back into solution by the addition of small portions of methanol and 10% aqueous acetic acid, after which the diafiltration was continued until no low molecular weight species were detected inside the cell (size-exclusion HPLC). The resulting solute was lyophilized to obtain the products as light orange powders. G-6-TEMPO-198: C 53.72, H 8.56, N 16.10. MALDI-TOF mass spectra: number average MW 101000; weight average MW 102000. G-6-TEMPO-80: C 49.56, H 8.13, N 16.75. MALDI-TOF mass spectra: number average MW 71000; weight average MW 72000.

Reoxidation of TEMPOL-H by spin-labeled dendrimers: The oxidation of TEMPOL-H (1 mM) to TEMPOL by TEMPO-conjugated dendrimer in the presence of 50 μM dendrimer was performed in Ar-saturated water (G-6-TEMPO-198) or Ar-saturated PBS (G-6-TEMPO-80 and G-6), and measured as a function of time. The plotted signal intensity due to the reoxidized TEMPOL-H (divided by gain, see Figure 1) was adjusted by subtracting the signal intensities of blank solutions of G-6-TEMPO-198 or G-6-TEMPO-80.

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Photochemical Carbonylation of Ethane under Supercritical Conditions**

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The photochemical carbonylation of hydrocarbons and aromatic compounds [Eq. (1)] by rhodium catalysts of the general formula [Rh(CO)L_2Cl] (where L = PMe_3, PPh_3) is

(1)

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