Direct solid-phase synthesis and fluorescence labeling of large, monodisperse mannosylated dendrons in a peptide synthesizer†

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Fluorophore-labeled glycodendrimers have potential use in the study of carbohydrate–protein interactions by fluorescence spectroscopy and imaging methods. The current solution-phase methods for preparation of such glycoconjugates are labour intensive. On the other hand, the intrinsically more efficient solid-phase methods have been explored only at low generations. Herein we disclose a direct, expedient glycodendrimer synthesis from commercially available or easily prepared building blocks by machine-assisted solid-phase peptide synthesis (SPPS). Large, monodisperse 4th- and 5th-generation polylysine dendrons are prepared and capped with 16 and 32 mannose residues, respectively, in a single synthetic operation. Incorporation of a C-terminal lysine residue in the 4th-generation dendron allows fluorescence labelling with a number of common labels on resin, in organic solvent or in aqueous buffer, as required. A single HPLC purification is sufficient in all cases to obtain a homogeneous sample. The monodispersity of the glycodendrons is confirmed by MALDI-TOF. FITC-labeled 4th-generation glycodendron is an excellent probe for the imaging studies of mannose-receptor-mediated entry of into dendritic cells by confocal fluorescence microscopy.

Introduction

Studies of carbohydrate–protein interactions,[1–3] a cornerstone in glycobiology, have resulted in the design of glycoconjugates for a variety of biomedical applications in drug discovery and delivery, vaccine design, biosensing and diagnostics.[4,5] For example, mannose receptors,[6,7] C-type lectins residing on the surface of dendritic cells—antigen presenting cells involved in both innate and adaptive immune responses[8,9]—interact with pathogens displaying polyvalent glycan chains composed of D-mannose, *N*-acetyl-D-glucosamine and L-fucose. After pathogen capture, internalization and digestion, peptide fragments are expressed on major histological complex (MHC) molecules.[10–12] T-cell-specific immune response is thereby initiated. This sequence of biologic events could be exploited for vaccine design: decoration of antigens with mannose residues in a variety of topologies leads to a significant increase of immunogenicity by mannose-receptor-mediated uptake.[13–19]

The intrinsic low affinity of a single protein–monosaccharide interaction is overcome in nature by multivalency, leading to significant binding enhancement per carbohydrate unit (cluster glycoside effect).[20] Therefore, application-oriented research in the field of carbohydrate–protein interactions is underpinned by synthesis of the large, highly multivalent glycoconjugates that display the necessary high binding efficiencies. Considerable effort has been devoted to preparation of numerous cluster glycosides and studies of their binding affinities in a variety of assays.[20,21] Due to their roughly spherical shape and well defined structure, carbohydrate-terminated glycodendrimers[22–24] in particular have received considerable attention. Poly-L-lysine dendrons, used earlier for the synthesis of multiple antigen peptides,[25,26] have also become one of the most popular glycodendrimer scaffolds for synthesis both in solution[27–30] and on solid supports.[16,31–36] Large (8 carbohydrate units or more) glycosylated polylysine dendrimers are among the most potent inhibitors to date: Lee and coworkers measured $IC_{50} = 0.9$ nM for the inhibition of adhesion of type 1 fimbriated *Echerichia coli* (a urinary tract pathogen) by a G4-polylysine dendron bearing 16 α-mannoside residues, a 12 500-fold decrease compared to α-methylmannoside;[37] the group of Roy determined $IC_{50} = 1.8$ μM for binding of a polylysine dendrimer carrying 16 mannose residues to the pea lectin, 2139 times lower than α-methylmannoside.[36]

Fluorescence spectroscopy[38,39] is one of the most important methods for biophysical characterization of biomolecular structure and dynamics. In addition, high sensitivity, low intrinsic background and availability of a wide range of commercial hardware have resulted in the proliferation of fluorescence-based bioimaging techniques, and reports of their applications have burgeoned in recent years.[40–44] For these applications, labeling of a biological target of interest with a fluorophore, most often an organic dye, is required.[45,46] Despite the potential of fluorescently labeled glycodendrimers to be used as probes to study carbohydrate–protein interactions in general, and the mannose-receptor-mediated uptake in particular, limited attention

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has been devoted to the preparation of such glycoconjugates. Typically, a glycodendrimer or a glycocluster is prepared by multi-step synthesis, purified and labeled with a fluorophore followed by additional purification of the resulting fluorescent conjugate.[13,16,33,47–49] Such solution-phase methods are tedious and labour-intensive. In contrast, Fréchet and coworkers recently disclosed a direct preparation of a small mannose cluster on polyglycerol dendritic backbone in an oligonucleotide synthesizer starting from a solid support already labeled with a popular fluorophore, 5-fluorescein isothiocyanate (FITC).[50] However, the challenge of practical synthesis of large glycodendrimers labeled with a range of fluorophores in an expedient, user-friendly manner still remains unanswered. Herein we provide one solution to this problem by exploiting the speed of solid-phase synthesis for direct synthesis and fluorescence labeling of large mannosylated polylysine dendrons and demonstrating the use of one of the products as an imaging probe for mannose-receptor-mediated entry into dendritic cells.

Results and discussion

Dendrimers[51,52]—monodisperse, branched, tree-like cascade macromolecules—have emerged as novel materials for a variety of medical and biological applications.[53–56] The use of solid supports enhances the efficacy of dendrimer synthesis by allowing a large excess of reagents to be used in order to drive the reaction cycle to completion while the purification stage is reduced to simple washing.[57–63] As an additional design consideration, we sought to avoid the use of non-biological functional groups, which could lead to potentially toxic artifacts when incorproated into the final bioconjugate. Therefore, we considered using the well-developed, high-yielding and operationally simple peptide coupling chemistry to assemble non-toxic, non-immunogenic polylysine dendrimers to which the required number of mannosyl residues can be attached simultaneously *via* a 4-hydroxybutanoic acid linker,[14] a naturally occurring substance in mammals.

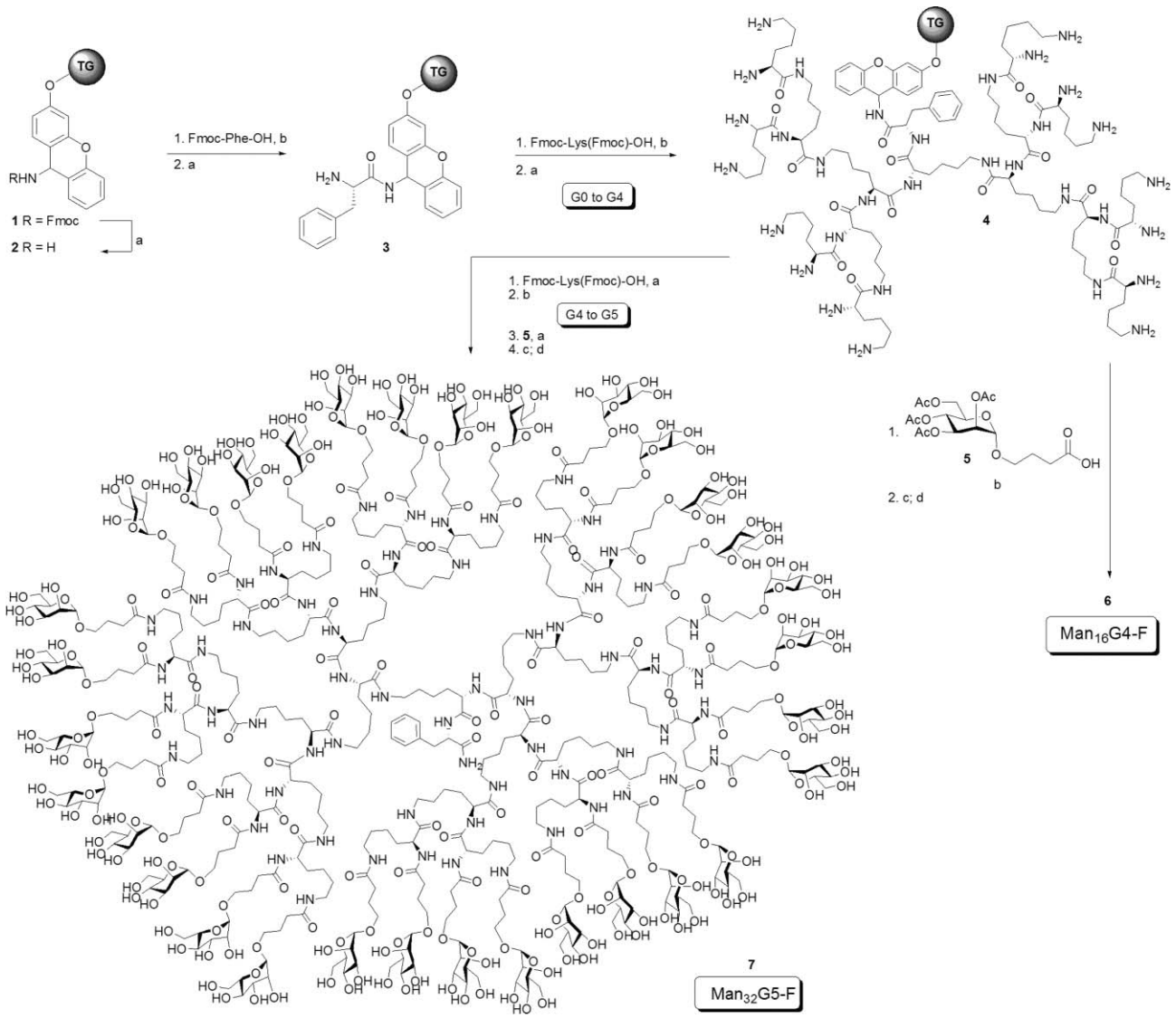
The synthesis of the polylysine dendrons was performed on commercial Tentagel resin preloaded with Fmoc-Sieber amide linker (**1**). The low loading level of the commercial resin (0.16 mmol g^{-1}) ensured that there would be sufficient space for the large 4th- and 5th-generation dendrons to be assembled without site-to-site interference.[64] At the same time, the mild cleavage conditions (10% TFA in CH_2Cl_2) would not cause significant acid-promoted side reactions. A single phenylalanine residue was introduced as a hydrophobic spacer after deprotection of the Fmoc group in **1** and coupling with Fmoc-Phe-OH (Scheme 1). After removal of the N-terminal Fmoc group, the 4th-generation polylysine dendron (G4; **4**) was extended from the N-terminus of the resin-immobilized Phe (**3**) using the commercially available Fmoc-Lys(Fmoc)-OH by reiterative peptide coupling and Fmoc-deprotection. Only ions arising from **4** as C-terminal carboxamide ($(H_2N)_{16}$G4-F-$CONH_2$) were observed by flow injection analysis (FIA) ESI-MS after cleavage from a small portion of the resin. The polymer-supported **4** was treated with an excess of activated, acetate-protected mannose–butanoic acid conjugate[14] (**5**) for 10 h, the dendron was liberated from the resin (10% TFA, CH_2Cl_2 in the presence of 1% triisopropylsilane as scavenger) and the acetyl groups were removed with NH_3 (2 M) in methanol over 18 h. The 4th-generation mannosylated dendron (Man_{16}G4-Phe, **6**) was isolated after semi-preparative reverse-phase (RP) HPLC.

Analogously, the resin-supported 4th-generation dendrimer (**4**) was successfully extended to the 5th-generation (Scheme 1). Complete generational growth was confirmed by FIA ESI-MS. The G5 dendron amine was treated with an excess of activated **5** and the corresponding G5 glycodendrimer (Man_{32}G5-F, **7**) was obtained after cleavage from the resin, acetate group removal and semipreparative RP-HPLC.

Encouraged by the success of the synthesis of large dendrons, we further adapted this methodology to the preparation of fluorescently labeled analogs by introducing a lysine residue at the focal point. The side chain amine protected by the very acid-labile 4-methyltrityl (Mtt) protecting group[65] can be used for labeling in conjunction with the considerably less acid-labile commercial Rink amide linker for maximum versatility. Rink amide Tentagel resin (**8**) was loaded with Fmoc-Lys(Mtt)-OH and the N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF (Scheme 2). Starting from **9**, the G3 dendron (**10**) was synthesized by reiterative treatment with PyBOP-preactivated Fmoc-Lys(Fmoc)-OH in a manner analogous to above. Further treatment with activated Fmoc-Lys(Fmoc)-OH afforded the G4 dendron. Completeness of both stages of dendrimer growth was confirmed by FIA ESI-MS. After mannosylation (**11**), treatment with 94% TFA results in simultaneous Mtt removal and dendrimer liberation (**12**). The fully deprotected G4 glycodendron (Man_{16}G4-K, **13**) was isolated after RP-HPLC purification.

The Mtt group can be removed at low TFA concentrations with minimal losses of the dendron and the newly revealed amine can be labeled on resin. Accordingly, treatment of the polymer-supported G4 dendron (**11**) with 3% TFA in CH_2Cl_2 led to complete Mtt group removal within 40 min (2 × 20 min each, Scheme 2, **14**). Lower concentrations of TFA were inefficient. The free amine in **16** was then treated with 5(6)-carboxytetramethylrhodamine (TAMRA)–HOBt ester (prepared by activation with PyBOP/NMM), or dansyl chloride. In both cases the labeling was found to be ≥90% by analytical HPLC, yielding dendrons **15** and **16**, respectively.

The protecting groups on the peripheral carbohydrate residues have a very strong effect on the solubility properties of the dendrons. Before the removal of the acetyl protecting groups, the dendrimers are insoluble in water but soluble in polar organic solvents (THF, DMF, acetonitrile, methanol), allowing labeling in these solvents. When the hydrophobic, acetyl-protected crude dendrimer (**12**) was exposed to 4-(1-pyrenyl)butanoic acid and EDCI in THF followed by treatment with methanolic ammonia, the pyrene-labeled G4 glycodendron **14** was isolated after a single semipreparative RP-HPLC purification. The glycodendron becomes soluble in water when fully deprotected. To illustrate labeling in aqueous media, the crude **13** was treated with 5-FITC in $NaHCO_3$ buffer (pH 9). Analytical HPLC showed approx. 85% labeling efficiency. A straightforward RP-HPLC purification afforded the labeled dendron (**18**) in yield comparable to the non-labeled analog. In all cases, a single purification step by semipreparative RP-HPLC was sufficient to obtain chromatographically homogeneous, fully deprotected G4 and G5 denrdons (Fig. 1). For fluorescence-labeled dendrons, the unlabeled **13**, excess fluorescence label and all byproducts were successfully removed during the HPLC purification stage (data not shown). In

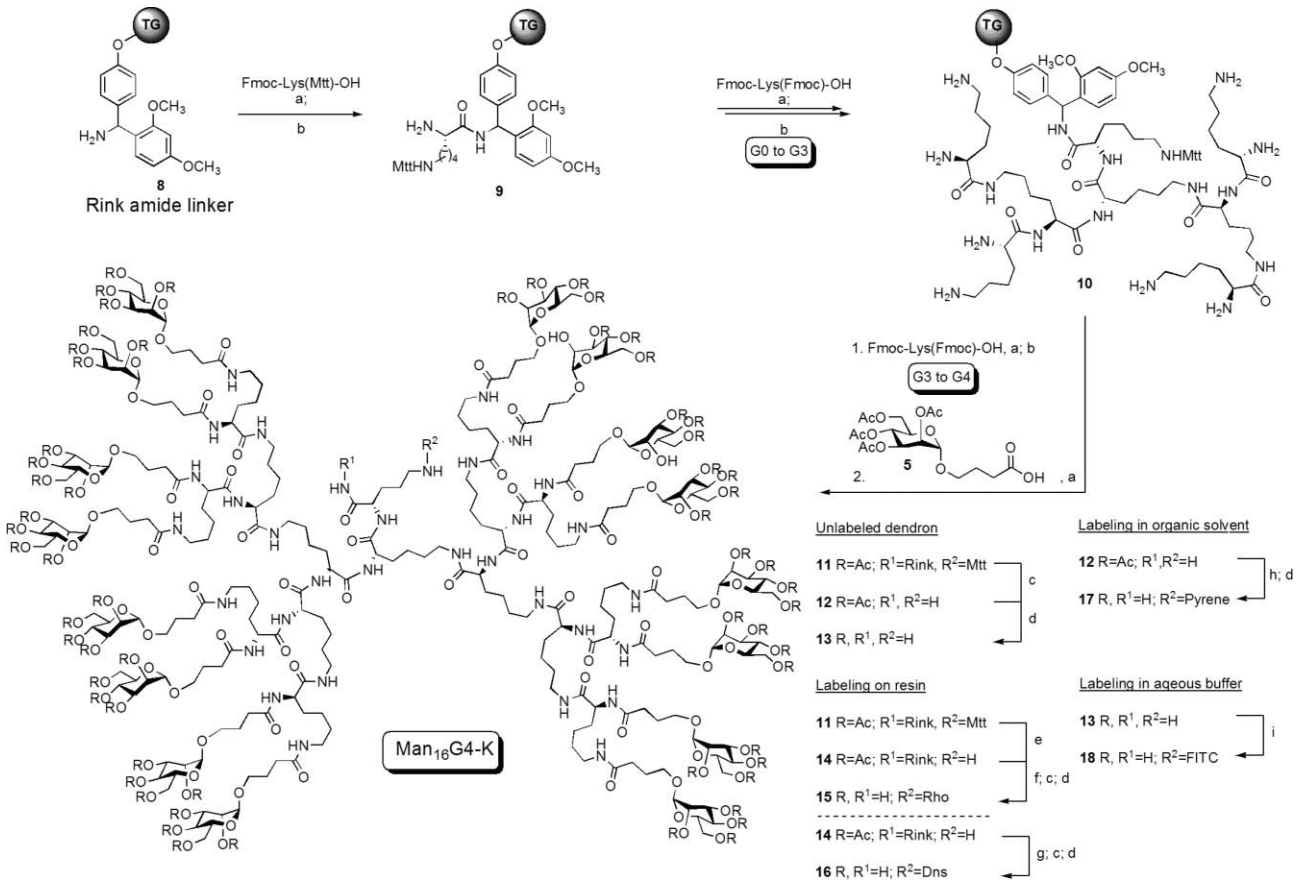


Scheme 1 Synthesis of mannosylated G4 and G5 dendrons. *Reagents and conditions*: a) 20% piperidine, DMF. b) PyBOP, *N*-methylmorpholine (NMM), DMF. c) 10% TFA, 1% *i*-Pr_3SiH, CH_2Cl_2, 3 h. d) 2 M NH_3, MeOH, 18 h.

the MALDI-TOF mass spectra of the dendrons, a fragmentation peak corresponding to the loss of a single mannose residue at *m*/*z* [M − 162] was also observed at both G4 and G5 levels (Fig. 2). The fluorescent dendrons showed enhanced ionizing ability and somewhat suppressed fragmentation in MALDI-TOF (Fig. 3). Even though the isolated yields for the glycodendrons vary, they are comparable with isolated yields for peptides prepared by Fmoc/*t*-Bu solid-phase peptide synthesis. *To the best of our knowledge, the G5 dendron (7), carrying 32 mannosyl residues, is the largest glycodendrimer (M_W 12.1 kDa) assembled on solid phase to date.*

UV-Vis and fluorescence spectra for the fluorescence labeled dendrons **15–18** were also recorded (Fig. 4). The absorbance and emission maxima observed were at the wavelengths expected.

Whereas some reports describing fluorescent glycodendrimer synthesis include no imaging experiments,[47,48,50] those that do have been exclusively focussed on the uptake of fluorescent glycodendrimers by cells expressing carbohydrate binding proteins on their surface. The group of Grandjean prepared tetra- and octavalent polylysine dendrimers terminated with mannose, galactose and two mannose mimetics, shikimic and quininic acid. The labelling was conducted in solution before the carbohydrate attachment by reaction of the N-terminal lysine with FITC.[33] The mannosylated dendrimer showed enhanced uptake by flow cytometry analysis in immature human monocyte-derived dendritic cells compared to the shikimic and quininic acid analogs, with the octavalent conjugate being twice as potent as the tetravalent. However, no further imaging studies were conducted.[15] The use of FITC- or 5(6)-carboxyfluorescein-labeled G2.5 PAMAM dendrimer carrying four *N*-Ac-D-glucosamine residues for the *in vitro* and *in vivo* imaging studies of uptake into NKR-P1A-positive lymphocytes was later reported by another group.[24] The dendrimers localized mainly in liver, kidney, spleen and cancer tissues in mice. We investigated the uptake of FITC-labeled G4 glycodendron **18** into dendritic cells as a model imaging application. Incubation at 4 °C with **18** (1 μM) led to very low fluorescence (data not



Scheme 2 Synthesis of G4 dendron with a C-terminal Lys and fluorescence labelling. *Reagents and conditions*: a) PyBOP, NMM, DMF. b) 20% piperidine, DMF. c) TFA–H_2O–*i*-Pr_3SiH, 94 : 3 : 3, 2.5 h. d) 2 M NH_3, MeOH, 18 h. d) 2 M NH_3, MeOH, 18 h. e) 3% TFA, 1% *i*-Pr_3SiH, CH_2Cl_2, 2 × 20 min. f) 5(6)-carboxytetramethylrhodamine, PyBOP, NMM, DMF, 10 h. g) dansyl chloride, *i*-Pr_2NEt, THF, 24 h. h) 4-(1-pyrenyl)butanoic acid, EDCI, cat. DMAP, THF–DMF = 10 : 1, 24 h. i) 5-FITC, aq. $NaHCO_3$ (pH 9)–CH_3CN, 24 h.

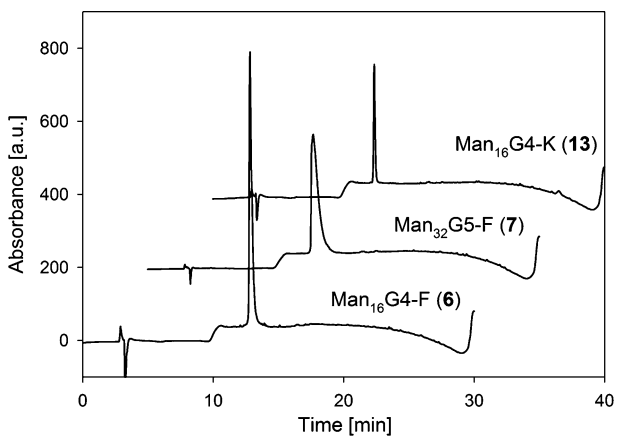


Fig. 1 Analytical RP-HPLC profiles of purified G4 and G5 dendrons. *Conditions*: Vydac C18 column, 5 to 95% CH_3CN in H_2O (containing 0.1% TFA) over 20 min.

shown). After warming up the cells to 37 °C, there was a 2 h incubation period before the confocal microscopy analysis in order to allow internalization (endocytosis) of the mannosyl dendrimer bound on mannose receptors at the cell surface. After the incubation, glycodendron **18** was observed in numerous vesicles inside dendritic cells (see ESI†). This suggests that the dendrimer initially binds to the cell membrane, followed by a mannose-receptor-mediated uptake at 37 °C. After direct incubation at 37 °C, similar pictures were obtained; a projection of images taken at different levels in the dendritic cells (0.3 μm) is presented in Fig. 5. The glycodendrimers persisted within the dendritic cells for at least 24 h (ESI). As a negative control for non-specific glycodendrimer–membrane receptor interaction or nonspecific pinocytosis,[8] **18** did not enter HeLa cells, which are devoid of mannose receptors (ESI). These results are very similar to previous studies by Roche *et al.* on linear oligolysine clusters capped with dimannoside or Lewis type oligosaccharides.[13,16]

Conclusions

In this work, we have presented the synthesis and fluorescence labelling of large, monodisperse mannosylated poly-L-lysine dendrons for imaging applications. The dendritic growth and mannosylation were accomplished in a single synthetic operation on a solid support, which allows a large excess of reagents to be used in order to drive the reaction to completion, thereby minimizing imperfections within the dendritic structure. Fourth- and fifth-generation mannosylated dendrons were prepared in a peptide synthesizer from commercial or easily prepared small building blocks. This method was especially designed for deployment in

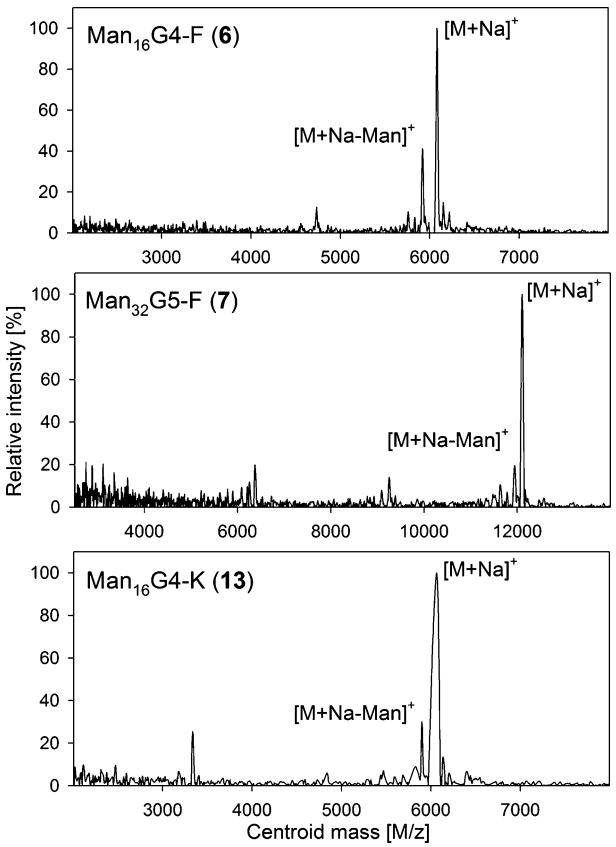


Fig. 2 MALDI-TOF mass spectra of purified G4 and G5 dendrons. Matrix: 2,3-dihydroxybenzoic acid.

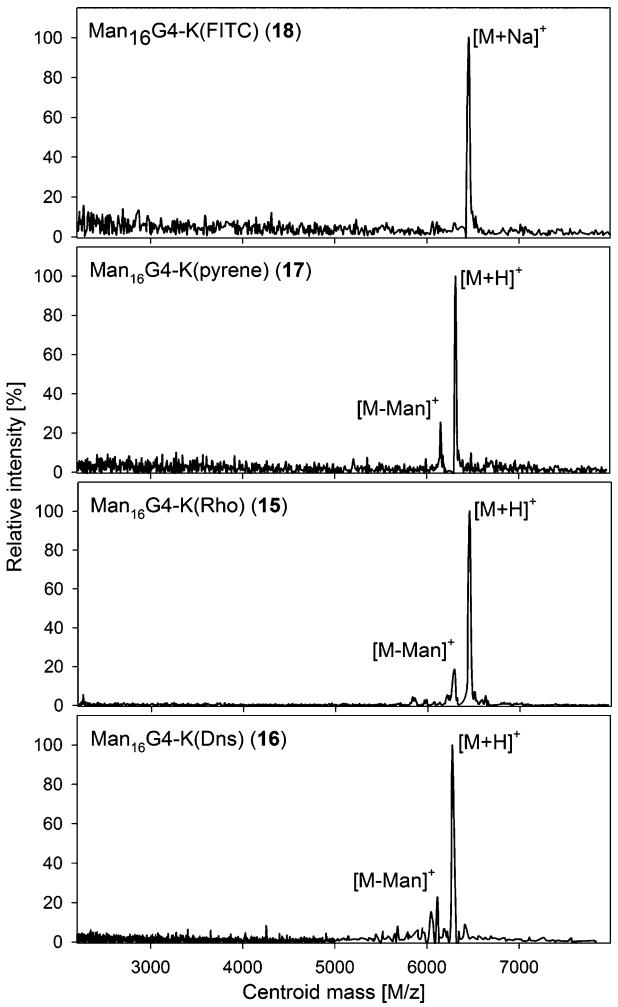


Fig. 3 MALDI-TOF MS of fluorescence-labelled G4 glycodendrons **15–18** (Scheme 2). Matrix: 2,3-dihydroxybenzoic acid.

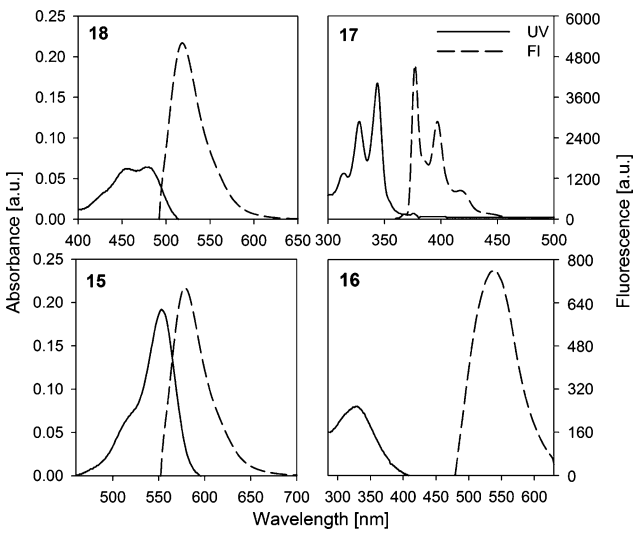


Fig. 4 UV-Vis absorption (solid line) and fluorescence emission (dotted line) of fluorescence-labelled G4 glycodendrons **15–18** (Scheme 2) in aqueous CH_3CN (10 vol%).

biological laboratories, and is easily integrated within the well-established manual or automated Fmoc/*t*-Bu solid-phase peptide synthesis methodology. The glycodendrons were obtained in a homogeneous state after a single HPLC purification step, as confirmed by HPLC and MALDI-TOF MS. The efficiency of this method is comparable to the standard peptide synthesis. The FITC-labeled 4th-generation dendron internalized into dendritic cells in a manner consistent with mannose-receptor-mediated uptake as monitored by confocal microscopy. Further applications of this synthetic methodology are ongoing in our laboratory.

Experimental

General

All reagents were from commercial sources and used without further purification, unless stated otherwise. All solvents were HPLC grade and used without further purification. Machine-assisted manual peptide synthesis was carried out in a Protein Technologies (Tucson, AZ, USA) PS3 synthesizer under N_2. The preactivated carboxylic acids were added manually, whereas solvent and deprotection solution dispensing and resin filtration were carried out by the hardware. DMF for solid-phase peptide synthesis was distilled in vacuum from anhydrous $MgSO_4$ prior to use. Fmoc-Phe-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Lys(Mtt)-OH, PyBOP, Rink amide resin (**8**, 0.20 mmol g^{-1}) and Fmoc-Sieber resin (**1**, 0.16 mmol g^{-1}) were purchased from Novabiochem. Amino acid activation solution (ACT) was 4.5% (vol/vol)

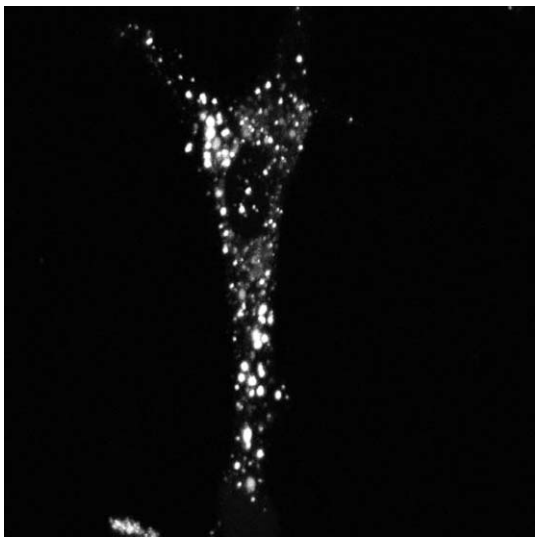


Fig. 5 Confocal microscopy analysis (projection of a *Z* series of 7 images) of the uptake of FITC-labelled G4 glycodendron (**18**) by dendritic cells. Incubation period: 2 h at 37 °C with **18** (1 μM).

N-methylmorpholine in DMF. Deprotection solution (DEP) was 20% (vol/vol) piperidine in DMF. HPLC was performed on an Agilent 1100 instrument equipped with a multiple-wavelength UV detector. Vydac C4 column (10 × 250 mm) was used for purifications, and Vydac monomeric C18 column (4.6 × 250 mm) for purity analysis of the final glycoconjugates. HPLC mobile phases were 5% (vol/vol) CH_3CN in water containing 0.1% (vol/vol) TFA (A), and 95% (vol/vol) CH_3CN in water containing 0.1% (vol/vol) TFA (B). HP ChemStation software was used for HPLC control and data management. All MALDI-TOF experiments were carried out on a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA). Volumes of 20 μL of matrix solution (20 mg mL^{-1} 2,3-dihydroxybenzoic acid in methanol–H_2O, 1 : 1 vol/vol) and 2 μL of the sample solution were combined.

Glycodendron synthesis and fluorescence labelling

Deprotection of Fmoc-Sieber amide resin (1). Fmoc-Sieber amide resin (0.16 mmol g^{-1}; 313 mg, 0.050 mmol, 1.0 equiv) was swollen in DMF for 1 h and washed with DMF (3 × 1 mL) and then treated with DEP solution (3 × 2 mL, 5 min each) and washing with DMF (3 × 2 mL).

Loading of the C-terminal Phe residue. The resin **2** was treated with Fmoc-Phe-OH (58 mg, 0.15 mmol, 3.0 equiv), preactivated with PyBOP (86 mg, 0.165 mmol, 3.3 equiv) in ACT solution (1.0 mL) for 1 h. After washing with DMF (1 mL), the coupling was repeated. Then, the resin was treated with acetic anhydride (0.25 mL) in ACT solution (2.0 mL) for 20 min and washed with DMF (3 × 2 mL). The N-terminal Fmoc group in **3** was removed as above.

Resin-supported $(H_2N)_{16}$-$K_8K_4K_2KF$ dendron (G4; 4). The resin **3** from the previous step was treated with Fmoc-Lys(Fmoc)-OH (89 mg, 0.15 mmol, 3.0 equiv) and PyBOP (86 mg, 0.165 mmol, 3.3 equiv) dissolved in ACT solution (0.50 mL) over 30 min, followed by washing with DMF (2 × 1 mL). The N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 5 min each), followed by washing with DMF (3 × 1 mL). Then, Fmoc-Lys(Fmoc)-OH (177 mg, 0.30 mmol, 6.0 equiv) and PyBOP (172 mg, 0.33 mmol, 6.6 equiv) dissolved in ACT solution (1.0 mL) were added to the resin, and the reaction mixture was agitated with a stream of N_2 and washed with DMF (2 × 1 mL). The N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 7.5 min each) and washed with DMF (3 × 1 mL). Fmoc-Lys(Fmoc)-OH (354 mg, 0.60 mmol, 12.0 equiv) and PyBOP (344 mg, 0.66 mmol, 13.2 equiv) dissolved in ACT solution (2.0 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 for 1 h. The resin was washed with DMF (2 × 1 mL), and the N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 10 min each) and washed with DMF (3 × 1 mL). Finally, Fmoc-Lys(Fmoc)-OH (708 mg, 1.20 mmol, 24.0 equiv) and PyBOP (688 mg, 1.32 mmol, 26.4 equiv) dissolved in ACT solution (3.0 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 for 1h. The resin was washed with DMF (2 × 1 mL) and the N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 10 min each), and washed with DMF (3 × 1 mL) and CH_2Cl_2 (3 × 10 mL). The G4 dendron resin **4** (427 mg) was obtained after filtration and drying in high vacuum.

Man_{16}-$K_8K_4K_2KF$-$CONH_2$ (G4 glycodendron; 6). The resin **4** (43 mg, 0.0050 mmol, 1.0 equiv) was suspended in DMF for 1 h and then washed with DMF (3 × 1 mL). A solution obtained by dissolving **5** (104 mg, 0.240 mmol, 48 equiv) and PyBOP (137 mg, 0.262 mmol, 53 equiv) in ACT solution (1.0 mL) was added and the reaction mixture was agitated with a stream of N_2 over 10 h. After washing with DMF (3 × 1 mL) and CH_2Cl_2 (3 × 10 mL), the resin was transferred into a flask and covered with TFA–*i*-Pr_3SiH–CH_2Cl_2 (10 : 1 : 90, vol/vol/vol, 2.0 mL total). After stirring for 3 h, the cleavage cocktail was removed in vacuum and the residue treated twice with hexane–ether (1 : 1, vol/vol, 10 mL), followed by dissolution of the crude dendrimer in CH_2Cl_2–MeOH (1 : 1,vol/vol, 20 mL), and filtering of the resin. The organic solvent was evaporated and the residue was dissolved in ammonia solution in methanol (2 M; 5.0 mL). After stirring overnight, the volatiles were removed in vacuum. The crude dendrimer was dissolved in aqueous CH_3CN (10 vol%, 25.0 mL). From an aliquot from this solution (4.0 mL), the pure G4 glycodendron **6** (2.5 mg) was isolated after semipreparative RP-HPLC and lyophilization. MALDI-TOF-MS: $[M + Na]^+$ calcd. for $C_{259}H_{448}N_{32}NaO_{128}$: 6081.5. Found 6081.6.

Man_{32}-$K_{16}K_8K_4K_2KF$-$CONH_2$ (G5 glycodendron; 7). The resin **4** (43 mg, 0.0050 mmol, 1.0 equiv) was suspended in DMF for 1 h and then washed with DMF (3 × 1 mL). Fmoc-Lys(Fmoc)-OH (142 mg, 0.24 mmol, 48 equiv) and PyBOP (137 mg, 0.26 mmol, 53 equiv) dissolved in ACT solution (1.0 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 for 1 h. The resin was washed with DMF (2 × 1 mL) and the N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 10 min each), and washed with DMF (3 × 1 mL). To the G5 resin thus obtained, a solution obtained by dissolving **5** (208 mg, 0.480 mmol, 96 equiv) and PyBOP (274 mg, 0.53 mmol, 106 equiv) in ACT solution (2.0 mL) was added and the reaction mixture was agitated with a stream of N_2 over 10 h. After washing with DMF (3 × 1 mL) and CH_2Cl_2, (3 × 10 mL), the product was cleaved

from the resin and the acetate groups were deprotected as described for compound **4**. The crude dendrimer was dissolved in aqueous CH_3CN (10 vol%, 25.0 mL). From an aliquot from this solution (5.0 mL), the G5 glycodendron **7** (6.65 mg) was isolated after semipreparative RP-HPLC and lyophilization. MALDI-TOF-MS: $[M + Na]^+$ calcd. for $C_{515}H_{896}N_{64}NaO_{256}$: 12 103.9. Found 12 104.5.

Loading of the C-terminal Lys(Mtt) residue. From Rink amide resin (**8**) (0.21 mmol g^{-1}; 475 mg, 0.1 mmol, 1.0 equiv) and Fmoc-Lys(Mtt)-OH (188 mg, 0.3 mmol, 3.0 equiv) activated with PyBOP (172 mg, 0.33 mg, 3.3 equiv) in ACT solution (1.5 mL), the resin **9** was obtained as described for compound **3**.

Resin-supported $(H_2N)_8$-K_4K_2KK dendron (G3; 10). The resin **9** from the previous step was treated with Fmoc-Lys(Fmoc)-OH (177 mg, 0.30 mmol, 3.0 equiv) and PyBOP (172 mg, 0.33 mmol, 3.3 equiv) dissolved in ACT solution (1.5 mL) over 30 min, followed by washing with DMF (2 × 1 mL). The N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 5 min each), followed by washing with DMF (3 × 1 mL). Then, Fmoc-Lys(Fmoc)-OH (354 mg, 0.30 mmol, 6.0 equiv) and PyBOP (344 mg, 0.33 mmol, 6.6 equiv) dissolved in ACT solution (2.5 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 and washed with DMF (2 × 1 mL). The N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 7.5 min each) and washed with DMF (3 × 1 mL). Fmoc-Lys(Fmoc)-OH (708 mg, 1.20 mmol, 12.0 equiv) and PyBOP (688 mg, 1.32 mmol, 13.2 equiv) dissolved in ACT solution (4.0 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 for 1 h. The resin was washed with DMF (2 × 1 mL) and the N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 10 min each), washed with DMF (3 × 1 mL) and then with CH_2Cl_2 (3 × 10 mL). The resin-immobilized G3 dendron **10** (588 mg) was obtained after filtration and drying in high vacuum.

Man_{16}-$K_8K_4K_2KK(NH_2)$-$CONH_2$ (G4 glycodendron; 13). The resin **10** (30 mg, 0.0050 mmol, 1.0 equiv) was suspended in DMF for 1 h and then washed with DMF (3 × 1 mL). Fmoc-Lys(Fmoc)-OH (71 mg, 0.12 mmol, 24.0 equiv) and PyBOP (69 mg, 0.132 mmol, 26.4 equiv) dissolved in ACT solution (0.50 mL) were added to the resin and the reaction mixture was agitated with a stream of N_2 for 1 h. The resin was washed with DMF (2 × 1 mL) and the N-terminal Fmoc groups were deprotected with DEP solution (2 × 1 mL, 10 min each), and washed with DMF (3 × 1 mL). To the G4 resin obtained, a solution obtained by dissolving **5** (104 mg, 0.24 mmol, 48 equiv) and PyBOP (137 mg, 0.53 mmol, 53 equiv) in ACT solution (1.0 mL) was added and the reaction mixture was agitated with a stream of N_2 over 10 h. After washing with DMF (3 × 1 mL) and CH_2Cl_2 (3 × 10 mL), the glycodendron was cleaved from the resin **11** using TFA–H_2O–*i*-Pr_3SiH (94 : 3 : 3, vol/vol/vol, 2.0 mL total volume) over 2.5 h. The acetate groups in **12** were deprotected with ammonia in methanol (2 M; 4.0 mL) for 18 h. The crude dendrimer was dissolved in aqueous CH_3CN (10 vol%, 25.0 mL). From an aliquot from this solution (5.0 mL), the pure G4 glycodendron **13** (1.1 mg) was isolated after semipreparative RP-HPLC and lyophilization. MALDI-TOF-MS: $[M + Na]^+$ calcd. for $C_{256}H_{451}N_{33}NaO_{128}$: 6062.5. Found: 6062.7.

Man_{16}-$K_8K_4K_2KK(Rho)$-$CONH_2$ (15). The acetyl-protected polymer-supported G4 dendron was prepared from **10** (90 mg, 0.015 mmol, 1 equiv) as described for compound **13** above. The G4 dendritic growth level was attained using Fmoc-Lys(Fmoc)-OH (212 mg, 0.345 mmol, 24 equiv) activated with PyBOP (211 mg, 0.405 mmol, 27 equiv) in ACT solution (2.0 mL). The fully protected, resin-supported glycodendron **11** (183 mg) was obtained after treatment a solution obtained by dissolvng **5** (313 mg, 0.72 mmol, 48 equiv) and PyBOP (421 mg, 0.81 mmol, 53 equiv) in ACT solution (3.5 mL). For Mtt deprotection, an aliquot from the resin **11** (61 mg, 0.0050 mmol, 1 equiv) was treated with CH_2Cl_2 containing TFA (3 vol%) and *i*-Pr_3SiH (1 vol%; 2 × 20 min, 3 mL each). The resin was washed with CH_2Cl_2 (5 × 1 mL), DMF (5 × 1 mL) and then with ACT solution (5 × 1 mL) to recover the side-chain amine as free base. The side-chain deprotected glycodendron **14** was suspended in a solution obtained by dissolving 5(6)-carboxytetramethylrhodamine (9.3 mg, 0.020 mmol, 4 equiv) and PyBOP (11.5 mg, 0.022 mmol, 4.4 equiv) in ACT (0.50 mL) and the reaction mixture was agitated with a stream of N_2 over 10 h. Cleavage and acetate deprotection were performed as described for compound **13**. The crude dendron was dissolved in aqueous CH_3CN (10 vol%, 25.0 mL). From an aliquot from this solution (7.25 mL), the pure Rho-labeled glycodendron **15** (3.4 mg) was isolated after semipreparative RP-HPLC and lyophilization. MALDI-TOF-MS: $[M + H]^+$ calcd. for $C_{281}H_{472}N_{35}O_{132}$: 6452.9. Found 6453.6.

Man_{16}-$K_8K_4K_2KK(Dns)$-$CONH_2$ (16). The side-chain deprotected glycodendrimer **14** (obtained as described above) was suspended in a solution of dansyl chloride (13.5 mg, 0.0500 mmol, 33 equiv) and *i*-Pr_2NEt (17.5 μL, 12.7 mg, 0.098 mmol, 65 equiv) in dry THF (1.50 mL) and the reaction mixture was shaken for 18 h. The crude dendron was obtained after cleavage from the resin and acetyl group removal as described for compound **13** and was dissolved in aqueous CH_3CN (10 vol%, 25.0 mL). From an aliquot from this solution (7.00 mL), the pure Dns-labeled glycodendron **16** (3.2 mg) was isolated after semipreparative RP-HPLC and lyophilization. MALDI-TOF-MS: $[M + H]^+$ calcd. for $C_{268}H_{463}N_{34}O_{130}S$: 6273.8. Found: 6274.6.

Man_{16}-$K_8K_4K_2KK(pyrene)$-$CONH_2$ (17). From **10** (30 mg, 0.0050 mmol, 1.0 equiv), crude acetyl-protected G4 dendron **12** was prepared was described for compound **13** above. The whole amount of **12** (12 mg) and 4-(pyrene-1-yl)butanoic acid (1.45 mg, 0.0050 mmol, 1.0 equiv) were dissolved in dry THF (0.85 mL). A solution containing 4-dimethylaminopyridine (DMAP) (0.03 mg, 0.25 μmol, 5 mol%) in dry THF (50 μL) was mixed with a solution of EDCI (1.15 mg, 0.0060 mmol, 1.2 equiv) in DMF (100 μL). The two solutions from above were mixed (THF–DMF = 10 : 1, v/v, total volume 1.0 mL) and shaken over 24 h. The volatiles were removed under reduced pressure and the residue dissolved in ethyl acetate (15 mL), washed with HCl (0.1 M), aqueous $NaHCO_3$

Table 1 UV-Vis spectroscopy. Scan speed: 100 nm min^{-1}

Dendron	Concentration/μM	Scan range/nm
18	11.7	400–520
17	24.8	300–500
15	7.9	400–500
16	37.6	285–410

Table 2 Fluorescence spectroscopy (Ex—excitation; Em—emission). Scan speed: 300 nm min^{-1}

Dendron	Concentration/μM	Excitation/nm	Scan range/nm	Spectral bandwidth/nm	
				Ex	Em
18	1.17	480	500–650	10	10
17	2.48	344	310–480	5	2.5
15	0.79	550	500–700	5	2.5
16	3.76	325	285–410	10	10

(0.1 M, 2 × 5 mL), dried ($MgSO_4$) and evaporated to dryness. The residue was dissolved in ammonia in methanol (2 M; 5.0 mL) and stirred over 24 h. The volatiles were removed in vacuum and the residue diluted with aqueous CH_3CN (10 vol%, 25.0 mL) From an aliquot from this solution (11.25 mL), the pyrene-labeled glycodendron **17** (0.85 mg) was isolated after semipreparative RP-HPLC purification and lyophilization. MALDI-TOF-MS: $[M + H]^+$ calcd. for $C_{276}H_{465}N_{33}O_{129}$: 6310.8. Found: 6311.7.

Man_{16}-$K_8K_4K_2KK$(FITC)-$CONH_2$ (18). The crude glycodendron **13**, prepared from the resin **10** (30 mg, 0.0050 mmol, 1.0 equiv) as described above, was dissolved in a mixture of aqueous $NaHCO_3$ (0.1 M, pH = 9.0; 10.0 mL) and CH_3CN (2 mL). A solution of 5-FITC (9.7 mg, 0.025 mmol, 5.0 equiv) in CH_3CN (0.50 mL) was added and the mixture was shaken in the dark for 24 h. The base was quenched with HCl (1 M, 1 mL) and diluted to 20.0 mL. From an aliquot from this solution (5.0 mL), the FITC-labeled glycodendron **18** (0.70 mg) was isolated after semipreparative RP-HPLC purification and lyophilization. MALDI-TOF-MS: $[M + Na]^+$ calcd. for $C_{277}H_{463}N_{34}NaO_{133}S$: 6452.8. Found: 6452.9.

UV-Vis and fluorescence spectroscopy

Using matched cells of 1 cm path length, all UV measurements were made on a Hitachi double beam spectrophotometer model U-2001, and fluorescence were performed on a Hitachi Fluorescence Spectrophotometer model F-2500, at ambient temperature in aqueous CH_3CN (10 vol%). The concentrations and parameters used were shown in Tables 1 and 2.

Confocal microscopy analysis of glycodendrimer entry into dendritic cells

DC-IL-13 (DC stands for 'dendritic cells", IL-13 stands for 'Interleukin-13") obtained from monocytes isolated by elutriation from peripheral blood mononuclear cells differentiated in the presence of GM-CSF and IL-13[66] were provided by IDM (Immuno Designed Molecules, Paris, France) in frozen vials, were plated on a cover slip after thawing and cultured in Aim-V medium supplemented with 500 units mL^{-1} GM-CSF and 50 ng mL^{-1} IL-13. After incubation in the complete medium overnight, the cells were treated with Man_{16}G4-K(FITC) (**18**; 1 μM, 200 μL) as follows: 1) 1 h at 4 °C, followed by washing and incubation for 2 h at 37 °C; and 2) incubation at 37 °C for 2 h followed by washing. The cells were then fixed for 20 min in PBS containing 4% formaldehyde. Cover slips were mounted on slides in a PBS–glycerol mixture (1 : 1, vol/vol) containing 1% 1,4-diazabicyclo[2.2.2]octane as an anti-fading agent. Cell fluorescence was analyzed with a confocal imaging system MRC-1024 (Bio-Rad) equipped with a Nikon Opitphot epifluorescence microscope and a 60× planapo objective (numerical aperture 1.4). A Kr/Ar laser was tuned to produce a 488 nm beam. Images were recorded with a Kalman filter average of 5–10 images; sequential *Z* series collected (*Z*-step: 0.3 μm).

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