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Convergent Assembly and Surface Modification of Multifunctional Dendrimers by Three Consecutive Click Reactions

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Abstract

Multifunctional dendrimers bearing two or more surface functionalities have the promise to provide smart drug delivery devices that can for example combine tissue targeting and imaging or be directed more precisely to a specific tissue or cell type. We have developed a concise synthetic methodology for efficient dendrimer assembly and heterobifunctionalization based on three sequential azide-alkyne cycloadditions. The methodology is compatible with biologically important compounds rich in chemical functionalities such as peptides, carbohydrates and fluorescent tags. In the approach, a strain promoted azide-alkyne cycloaddition (SPAAC) between polyester dendrons modified at the focal point with an azido and 4-dibenzocyclooctynol (DIBO) moiety provided dendrimers bearing terminal and TMS-protected alkynes at the periphery. The terminal alkynes were outfitted with azido-modified polyethylene glycol (PEG) chains or galactosyl residues using Cu^{I} catalyzed azide-alkyne cycloadditions (CuAAC). Next, a one-pot TMS-deprotection and second click reaction of the resulting terminal alkyne with azido-containing compounds gave multifunctional dendrimers bearing complex biologically active moieties at the periphery.

Keywords

dendrimers; carbohydrates; peptides; synthetic methods; drug delivery; click chemistry

Introduction

Dendrimers are emerging as promising materials for the development of imaging devices and drug and gene delivery vehicles.[1] Attractive properties of dendrimers include chemical homogeneity, tunability of biodistribution and pharmacokinetics by regulating size and controlled degradation by judicious choice of dendrimer chemistry.[2] Furthermore, the typical architecture of dendrimers results in the formation of cavities, which can entrap pharmaceutically active substances.[3] Moreover, the surface of dendrimers can be modified by prodrugs, imaging modules such as fluorescent tags, CT and MRI contrast agents,[4] polyethylene glycol to increase water solubility and improve biocompatibility,[5] and by cell tissue targeting ligands such as folic acid or RGD peptides to increase therapeutic efficiency.[6] Surface modification of dendrimers with a targeting device benefits from high multivalent densities, which will strengthen ligand-receptor binding as a result of a cluster

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Supporting information is available.

effect.[7] A particularly attractive approach for surface modification of dendrimers is a Cu^{I} catalyzed 1,3-dipolar cycloaddition of azides with terminal alkynes (CuAAC) to give stable 1,2,3-triazoles.[8] CuAAC combines exceptional chemoselectivity with a lack of byproducts and high yields. It has been used to efficiently derivatize dendrimers with unprotected peptides,[9] carbohydrates,[10] and other complex compounds.[11]

It is to be expected that dendrimers modified by several different peripheral entities can combine functions such as tissue targeting and imaging or be directed more precisely to a specific tissue or cell type.[12] Usually, multifunctional dendrimers are prepared by a random chemical coupling reaction, which unfortunately leads to unwanted dispersity.[13] A more attractive approach uses dendritic molecules or polymers having two or more orthogonal functionalities or protecting groups.[14] In particular, azides or alkynes for CuAAC combined with hydroxyls for etherification[15] or aldehydes for hydrazone formation[16] have been successfully employed as sets of the orthogonal functionalities. Dendrimers have also been multifunctionalized using CuAAC in a sequential manner. In this approach, polyester dendrimers modified by mannoside-targeting moieties and coumarin fluorescent tags were prepared by starting with a dendrimer having peripheral alcohols and isopropylidene acetals.[17] The alcohols of the dendrimer could be modified by terminal alkynes, which could then be coupled with azide-modified coumarin. Removal of isopropylidene acetals gave alcohols and a repetition of alkyne formation and CuAAC led to the controlled introduction of peripheral mannosides. More recently, bifunctional[18] and trifunctional[19] dendrimers were constructed by click reaction followed by coupling of azide bearing dendron to the dendrimer core, thus enabling a surface modification by a second CuAAC.

Despite many attractive features of these methods, the limited chemoselectivity of conventional functional groups such as alcohol, amine, carboxylic acids and carbonyls and in some cases the relatively large number of chemical steps for orthogonal group installation, places restrictions on the type of functionality that can be attached to a dendritic surface.

We report here a versatile approach for selective surface modification of dendrimers by a strain-promoted alkyne-azide cycloaddition (SPAAC)[20] between two dendrons modified by a focal dibenzocyclooctyne or azide and having peripheral alkynes or TMS-protected alkynes, respectively. This ligation exploits a selective reaction of a strained alkyne with an azide in the presence of terminal alkynes.[21] The terminal alkynes can, however, be selectively modified with an azide-containing moiety using a Cu^{I} catalyst. In a third step, a second type of surface functionality can be installed in a controlled manner by removal of the TMS-protecting groups followed by another CuAAC (Figure 1). The excellent chemoselectivity of SPAAC and CuAAC ensures that a wide variety of functionalities, such as biological relevant carbohydrates and peptides, can be attached in a controlled manner to the periphery of dendrimers.

Results and Discussion

First, we examined whether a strain-promoted alkyne-azide cycloaddition can be utilized for the ligation of two dendrons. Such a reaction is challenging due to steric hindrance at dendron focal points, which may render couplings inefficient leading to low yields and loss of expensive dendrons. Thus, we synthesized generation three- and four-dendrons **14**, **16**, **18** and **20** having a polyester dendritic framework based on 2,2-bis(hydroxymethyl) propionic acid (bis-MPA)[22] and bearing an azide or a 4-dibenzocyclooctynol (DIBO) moiety[20c] at the focal point (Scheme 1). A polyester framework was selected because of its intrinsic biodegradability[23] and good solubility in organic solvents.[24] DIBO was used because it

reacts fast with azido-containing compounds in the absence of a metal catalyst, can be prepared by a simple synthetic approach, is nontoxic and can easily be attached to a variety of probes.

Dendron synthesis started with a coupling of 2-(2-(2-azidoethoxy)ethoxy)ethanamine[25] (**3**) with isopropylidene protected bis-MPA anhydride **1**[22] in the presence of pyridine and dimethyaminopyridine (DMAP) in DCM to give amide **10**, which was treated with Dowex H^+ resin in MeOH to remove the isopropylidene acetals and reveal alcohols. Each subsequent generation was introduced by reaction of hydroxyls with anhydride **1** followed by removal of the isopropylidene protecting groups. In this way, polyester dendrons **11**, **12** and **13** were synthesized having masked alcohols at the periphery and an azide at the focal point. After deprotection of the isopropylidene acetals of **12** and **13**, peripheral alkynes were introduced by treatment with pent-4-ynoic anhydride (**4**) to give **14** and **18**, respectively. Alternatively, treatment of **11** with Dowex-H^+ followed by reaction of the resulting alcohols with 5-(trimethylsilyl)pent-4-ynoic anhydride (**5**) gave **21**, which has alkynes protected by trimethylsilyl (TMS) groups. All transformation proceeded in high yield leaving the important azide moiety at the focal point of the dendrons intact.

The azido-containing dendrons **14** and **18** were the starting material for the preparation of the DIBO containing derivatives **16** and **20**, respectively. Thus, reduction of the azides of **14** and **18** with trimethylphosphine in a mixture of THF and water gave the corresponding amines **15** and **19**, which were immediately treated with the activated carbonate of DIBO (**2**) [20c] to provide the requisite compounds **16** and **20** in yields of 89 and 93%, respectively.

Having azide- and DIBO-modified dendrons at hand, attention was focused on SPAAC-mediated ligation[26] of these derivatives. Gratifyingly, reaction of the G4 dendrons **18** and **20** in THF at room temperature proceeded smoothly and gave, after a reaction time of 24 h, symmetrical dendrimer **22** in a yield of 93% (Scheme 2). Also, the focal DIBO moiety of **16** could be employed for installing a fluorescent probe and reaction with azido-modified fluorescein **7** gave derivative **17**. Importantly, the copper free coupling required only a stoichiometric quantity of dendrons.

In the presence of a catalytic amount of $CuSO_4$, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) and sodium ascorbate in a mixture of THF and water, the alkynes of **17** and **22** could be reacted with azido-containing compounds (see supporting information for modification of **17** and G3-G3 symmetrical dendrimer **32**). For example, peripheral modified dendrimers **23** and **24** were obtained in good yields by reaction of **22** with 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanol[27] (**6**) and azido-containing galactoside[28] **9**, respectively (Scheme 2). The ^{1}H-NMR spectra of the CuAAC products showed characteristic triazole signals (8 ppm), integration of which along with the unique CH_2–triazole signals (4.5 ppm) of the galactosyl or tetraethyleneglycol residues, yielded in each case thirty-two triazole residues per dendrimer molecule (Figure 2). Complete surface derivatization of compound **20** was additionally confirmed by quantitative sugar analysis (31.8±0.2 galactosyl residues).

Next, we explored whether SPAAC can facilitate coupling of two dendrons having either terminal or TMS-protected alkynes to give dendrimers that can be modified in a controlled manner by two different surface entities. In this respect, a number of studies have shown that CuAAC can be performed without affecting a TMS-protected alkyne.[29] However, the TMS protecting group can easily be removed by reagents such as tetrabutylammonium fluoride (TBAF) or silver salts and the resulting terminal alkyne be used in a subsequent click reaction. The challenge for using this methodology for dendrimer modification is that multiple TMS-protected alkynes have to stay intact during dendrimer assembly and the first

CuAAC. Thus, SPAAC mediated coupling of **16** with **21** in THF for 5 h gave clean formation of the asymmetrical dendrimer **25**, which has terminal and TMS-protected alkynes at its periphery (Scheme 3). Next, **25** was subjected to azido-containing tetraethyleneglycol **6** in the presence of a catalytic amount of CuI and *N*,*N*-diisopropylethylamine (DIPEA). Under these conditions the terminal alkynes underwent a clean cycloaddition and after a reaction time of 4 h and purification by LH-20 size exclusion column chromatography, dendrimer **26** was obtained in a yield of 87%. Similarly, a reaction between asymmetrical dendrimer **25** and unprotected galactoside **9** afforded glycodendrimer **27** in a yield of 75%. Careful analysis of the structures of **25**, **26** and **27** by ^{1}H-NMR and MALDI ToF revealed that the TMS-protected alkynes had remained intact during the SPAAC and CuAAC click reactions. Partial desilylation was, however, observed when a combination of $CuSO_4$ and sodium ascorbate was used for the CuAAC.

Previously, we found that CuF_2 can efficiently unmask TMS-modified alkynes and promote cycloadditions with azides.[30] Fortunately, this protocol could be employed for the modification of **26** and reactions with RGD peptide **8** and galactoside **9** proceeded smoothly when methanol was used as a solvent at a temperature of 40°C, to give bifunctional dendrimers **28** and **29**, respectively. Bifunctional dendrimer **30** bearing unprotected galactoside residues and RGD peptides was obtained in a similar manner by treatment of glycodendrimer **27** with peptide **8** with CuF_2 in methanol-water mixture at 40°C. The absence of characteristic TMS proton signals in the ^{1}H-NMR and correct integral areas of sugar and peptide protons indicated complete derivatization of dendrimers **26** and **27**.

Conclusion

We have developed a convenient approach for dendrimer assembly and peripheral functionalization using three consecutive azide-alkyne cycloadditions. Strain promoted azide-alkyne cycloaddition was established as an effective and chemoselective method for coupling of dendrons to give symmetrical and asymmetrical dendrimers bearing alkynes on the periphery. Differentiated terminal and TMS-protected peripheral alkynes were efficiently modified with different combinations of model PEG, galactosyl and peptide-azides, bearing no protecting groups. The methodology is compatible with compounds that are rich in chemical functionalities such as peptides, carbohydrates and fluorescent tags. Furthermore only three consecutive steps are required for dendron coupling and installment of two-different surface entities. Recently, photo-,[31] thiol-ene,[32] and strain-promoted alkyne-nitrone[33] click reactions have been introduced, which also display excellent chemoselectivity and it is to be expected that integration of these reactions in the approach reported here will give easy access to even more complex dendritic structures.

Experimental Section

General methods

All chemicals and dry solvents were purchased from Sigma-Aldrich unless stated otherwise. All esterification, amidation, Staudinger reduction, $CuSO_4$ and CuI mediated reactions were carried out under an atmosphere of argon. Reactions were performed at room temperature (20–22 °C), unless stated otherwise. Reactions were monitored by Thin Layer Chromatography (TLC) using aluminum backed silica gel 60 (F254) plates, visualized using UV (254 nm) and potassium permanganate and cerium molybdate dips as appropriate. Flash chromatography was carried out using silica gel G60 (SiliCycle, 60–200μm 60 Å) as the stationary phase. Solid-Phase Peptide Synthesis (SPPS) was performed on a Applied Biosystems, ABI 433A peptide synthesizer equipped with UV-detector using $N^{á}$-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tertamethyl hexafluorophosphate (HBTU) / 1-hydroxybenzotriazole (HOBt) as the activating reagents.

Reverse Phase HPLC was performed on an Agilent 1200 series system equipped with an automated injector, UV-detector, fraction-collector and Agilent Zorbax Eclipse XD8-C18 column (5 μm, 9.4 × 250 mm). NMR spectra were recorded on Varian Mercury (300, 500 MHz) spectrometers at 25°C. Chemical shifts are reported in *ä* units, parts per million (ppm) downfield from TMS, spectra are referenced by solvent signals. Coupling constants (*J*) are measured in Hertz (Hz) and are unadjusted. Splitting patterns are designed as follows: s – singlet, d – doublet, t – triplet, dd – doublet of doublets, dt – doublet of triplets, td – triplet of doublets, m – multiplet, br – broad. Mass spectra were obtained using MALDI-ToF instruments (Applied Biosystems 4700 Proteomics Analyzer, Bruker Microflex LT Mass Spectrometer) with 2,5-dihydroxybenzoic acid or á-cyano-4-hydroxycinnamic acid as a matrix. Sugar analysis was performed on DIONEX ICS-3000 HPAEC chromatograph using deionizer water and 200 mM NaOH as an eluent.

General procedure for the synthesis of dendrons 10, 11, 12, 13, 14, 18 and 21 by sequential isopropylidene acetal removal and ester formation

Dowex® 50WX8-200 H^+ ion-exchange resin (2–4 g) was added to the solution of isopropylidene protected dendron (G1 to G4) in MeOH (10 mL) and the resulting suspension was stirred for 2–24 h at 50°C. The reaction mixture was filtered and the resin was washed with MeOH (3×10mL). The combined filtrates were concentrated under reduced pressure to give hydroxyl terminated dendron which was used in the next step without further purification. Hydroxyl terminated dendron or amine **3**, DMAP and pyridine were dissolved in DCM (5–10 mL). The mixture was cooled to 0°C and a solution of a suitable anhydride **1**, **4** or **5** in DCM (10–15 mL) was added to the mixture in small portions over 10 min. The reaction mixture was then allowed to warm to room temperature and stirred for 12–18 h. The solution was diluted with DCM (50–100 mL) washed with water (50–100 mL), sat. aq. $NaHCO_3$ (50–100 mL), sat aq. $CuSO_4$ (for anhydrides **1** and **5**, 50–100 mL) or 0.1 M HCl (for anhydride **4**, 2×50 mL) and brine (50–100 mL). The organic layer was dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel. Analytical data for dendrons **10**, **11**, **12**, **13**, **14**, **18** and **21** are reported in the supporting information.

General procedure for the installation of a cyclooctynol moiety for the preparation of 16 and 20

Azido-containing dendron (0.06 mmol) was dissolved in a mixture of THF and H_2O (9:1, v/v, 5 mL). A 1 M solution of trimethylphosphine in THF (0.61 mL, 0.61 mmol) was added to the solution and the resulting mixture was stirred for 3 h. The solution was concentrated *in vacuo* and coevaporated with toluene (3×10 mL). The resulting yellow residue and 11,12-didehydro-5,6-dihydrodibenzo[a,e]cycloocten-5-yl carbonic acid 4-nitrophenyl ester[20c] (**2**) (35 mg, 0.09 mmol) were dissolved in DMF (5 mL). DIPEA (0.04 mL, 0.24 mmol) was added to the solution and the resulting mixture was stirred for 48 h. Evaporation of the solvent under reduced pressure gave a residue, which was purified by silica gel column chromatography. Analytical data for dendrons **16** and **20** are reported in the supporting information.

General procedure for Cu-free ligation of dendrons to give 17, 22 and 25

Cyclooctynol-modified dendron and azido-containing dendron (or azide **7**) were dissolved in THF (10 μmol/mL) and the resulting mixture was stirred for 2–24 h. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography. Analytical data for dendrimers **17, 22** and **25** are reported in the supporting information.

General procedure for CuAAC reactions using $CuSO_4$ and Na ascorbate for the preparation of 23 and 24

Alkynylated dendrimer, azide **6** or **9** and tris[(1-benzyl-1H-1,2,3 -triazol-4-yl)methyl]amine (TBTA) were dissolved in THF (5 μmol/mL of dendrimer). 0.1 M Solution of (+)-sodium L-ascorbate and 0.1 M solution of $CuSO_4$ in water were added to the mixture. The reaction mixture was stirred for 18 h. Solvent was evaporated and the residue was purified via HPLC. Fractions of interest were combined and lyophilized. Analytical data for dendrimers **23** and **24** are reported in the supporting information.

General procedure for CuAAC reaction using CuI for the preparation of 26 and 27

Dendrimer **25**, azide **6** or **9**, CuI and DIPEA were dissolved in THF (0.01 mmol/mL). The reaction mixture was stirred for 4–20 h and the solvent was evaporated *in vacuo*. The residue was purified by SEC on Sephadex® LH-20 gel (MeOH:DCM, 1:1, v/v). Analytical data for dendrimer **26** are reported in the supporting information.

General procedure for CuF_2 mediated click reaction for the preparation of 28, 29 and 30

Dendrimer **26**, azide **9** or azido-peptide **8** and CuF_2 were dissolved in MeOH (4 μmol/mL). The reaction mixture was stirred at 40°C until completion of the reaction (monitored by MALDI-TOF MS). The solvent was evaporated and the residue was purified by HPLC to give after lyophilization of appropriate fractions the product. Analytical data for dendrimers **28** and **29** are reported in the supporting information.

Dendrimer 25 (isomers)

Prepared from G3 dendron **16** (36.9 mg, 20 μmol) and G2 dendron **21** (25.0 mg, 22 μmol) according to the general procedure for Cu-free ligation of dendrons. The reaction mixture was stirred for 5 h. Silica gel column chromatography (40% acetone in hexanes) gave dendrimer **25** (53 mg, 90%) as viscous oil: ^{1}H NMR (500 MHz, CD_6CO, 25°C, TMS): *ä*= 7.68–7.15 (m, 10 H, CH, NH), 6.55–6.46 (m, 1 H, NH), 6.22–5.97 (m, 1 H, CH), 4.65–4.56 (m, 2 H, CH_2), 4.34–4.28 (m, 40 H, CH_2), 4.01–3.79 (m, 2 H, CH_2), 3.79–2.79 (m, 22 H, CH_2), 2.60–2.46 (m, 48 H, CH_2), 2.36 (s, 8 H, CH), 1.37–1.28 (m, 30 H, CH_3), 0.11 ppm (s, 36 H, CH_3); MS (MALDI-TOF): *m/z*: calcd for $C_{151}H_{200}N_6O_{48}NaSi_4$: 3000.2 $[M+Na]^+$; found: 3001.3.

Dendrimer 27 (isomers)

Prepared from dendrimer **25** (15.0 mg, 5.0 μmol) using: 3-azidopropyl β-D-galactopyranoside (**9**) (21.0 mg, 80.0 μmol), CuI (0.8 mg, 4.5 μmol) and DIPEA (4 μL, 20.0 μmol) according to the general procedure for CuAAC reaction using CuI. The reaction mixture was stirred for 20 h. SEC purification gave **27** as transparent oil (19 mg, 75%): ^{1}H NMR (500 MHz, CD_3OD, 25°C, TMS): δ= 7.83 (s, 8 H, CH), 7.66–7.19 (m, 8 H, CH), 6.15–5.92 (m, 1 H, CH), 4.60–4.50 (m, 18 H, CH_2), 4.25–4.10 (m, 48 H, CH_2, CH), 4.01–3.24 (m, 88 H, CH_2, CH), 3.01–2.91 (m, 16 H, CH_2), 2.81–2.67 (m, 16 H, CH_2), 2.57–2.44 (m, 16 H, CH_2), 2.21–2.08 (m, 16 H, CH_2), 1.31–1.12 (m, 30 H, CH_3), 0.10 (s, 36 H, CH_3); MS (MALDI-TOF, most abundant mass): *m/z*: calcd for $C_{223}H_{336}N_{30}O_{96}Si_4Na$: 5108.1 $[M+Na]^+$; found: 5108.3.

Dendrimer 30 (isomers)

Dendrimer **27** (13.0 mg, 2.6 μmol), azido-RGD peptide **8** (7.5 mg, 15.4 μmol) and CuF_2 (2.1 mg, 20.8 μmol) were dissolved in MeOH:H_2O mixture 1:1 v/v (0.5 mL). The reaction mixture was stirred for 40 h at 40°C and the solvent was evaporated. The residue was purified by HPLC (t = 23.7 min) to give after lyophilization **30** (10.0 mg, 57%) as a white foam. ^{1}H NMR (500 MHz, D_2O, 25°C, TMS): δ= 7.81 (s, 12 H, CH), 7.59–7.11 (m, 8 H,

CH), 6.12–5.80 (m, 1 H, CH), 5.27 (s, 8 H, CH_2), 4.73 (dd, ^3J(H,H) = 7.2, 5.6 Hz, 4 H, CH), 4.52–4.43 (m, 18 H, CH_2), 4.37–3.84 (m, 84 H, C*H*$_2$, C*H*), 3.79–3.25 (m, 72 H, CH_2, CH), 3.17 (t, ^3J(H,H) = 6.8 Hz, 8 H, CH_2), 2.99–2.83 (m, 32 H, CH_2), 2.79–2.62 (m, 24 H, CH_2), 2.21–2.06 (m, 16 H, CH_2), 1.92–1.72 (m, 8 H, CH_2), 1.70–1.56 (m, 8 H, CH_2), 1.32–0.99 (m, 30 H, CH_3); MS (MALDI-TOF, MW, linear mode): *m/z*: calcd for $C_{275}H_{413}N_{74}O_{124}$ $[M+H]^+$ = 6739.6; found: 6720.7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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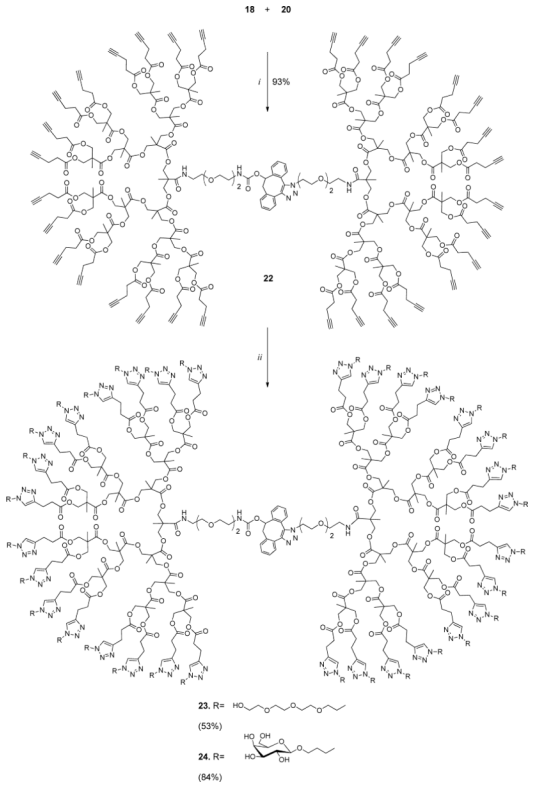
Figure 1.
General concept of multifunctional dendrimer synthesis by three consecutive click reactions.

Scheme 1.

a) Dendron building blocks. b) Azides for dendrimer derivatization. c) Synthesis of dendrons, introduction of dibenzocyclooctyne and copper free click reaction. Reaction conditions: *i.* isopropylidene-2,2-bis(methoxy)propionic anhydride (**1**), DMAP, Py, DCM, 0°C then RT, 12–18 h. *ii.* DOWEX® H^+ resin, MeOH, 50°C, 2–24 h. *iii.* pent-4-ynoic anhydride (**4**), DMAP, Py, DCM, 0°C then RT, 18 h. *iv.* 5-(trimethylsilyl)pent-4-ynoic anhydride (**5**), DMAP, Py, DCM, 0°C then RT, 18 h. *v.* PMe_3 10 eq., THF:H_2O, 9:1 v/v, 3 h; *vi*. **2**, DMF, DIPEA, 48 h; *vii*. **7**, THF, 2 h.



Scheme 2.

Dendrimer assembly via SPAAC, followed by CuAAC mediated derivatization. Reaction conditions: *i*. THF, 24 h; *ii*. **6** or **9**, $CuSO_4$, Na ascorbate, TBTA, THF:H_2O, 18 h.

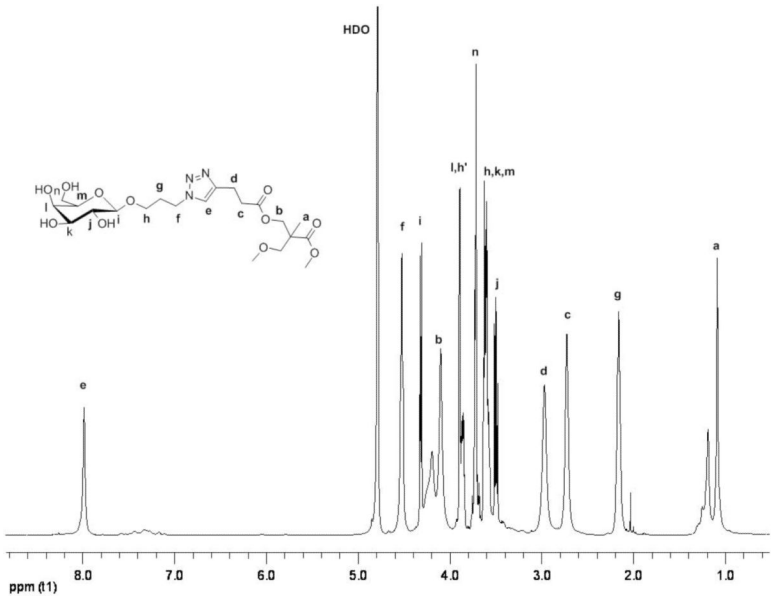
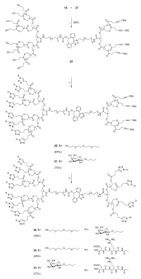


Figure 2.
^{1}H-NMR spectrum of glycodendrimer **24** (D_2O, 500 MHz).



Scheme 3.
Three consecutive AAC reactions leading to bifunctionalized dendrimers. Reaction conditions: *i.* THF, 5 h; *ii*. **6**, CuI, DIPEA, THF, 4 h; *iii*. **8** or **9**, CuF_2, MeOH or MeOH:H_2O, 40°C, 8–40 h.