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Convergent dendrimer synthesis by olefin metathesis and studies toward glycoconjugation.

Roberto Guizzardi, Mattia Vacchini, Carlo Santambrogio, Laura Cipolla*

Dept. of Biotechnology and Biosciences, University of Milano-Bicocca

Piazza della Scienza 2, 20126 Milano, Italy

* Corresponding author: Prof. Laura Cipolla, Department of Biotechnology and Biosciences, University of Milano-Bicocca, P.za della Scienza 2, 20126 Milano, Italy, Tel. +39 02 64483460; Fax: +39 02 64483565. E-mail: laura.cipolla@unimib.it
Abstract

The synthesis of novel hyperbranched monodisperse linear dendrimers, based on 2,2-bis-(hydroxymethyl)-propionic acid (bis-MPA), has been achieved by convergent metathesis-mediated coupling between the alkene-terminated focal point of bis-MPA dendrons. On their surface, dendrimers present 4, 8 and 16 functional groups. Glycodendrimers exposing multiple saccharide moieties have also been obtained. To the best of our knowledge, this is the first example of the use of metathesis for focal point coupling.

Keywords. Dendrimers, glycodendrimers, olefin metathesis, alkoxyamino-carbonyl chemistry
Introduction

Dendrimers are nano-sized macromolecules, featured with a highly branched structure, displaying an elevated number of functional groups on their surface, that can be exploited for further derivatisation with different kind of (bio)molecules. Given their peculiar structure and properties, dendrimers have been proposed for a variety of applications in the biomedical field, i.e. for drug and gene delivery, anti-cancer agents, magnetic resonance imaging contrast agents, photodynamic therapy, biosensors), or as scaffolds for light-harvesting, emission and amplification. Dendrimers can be synthesised mainly by two main approaches, referred to as convergent or divergent. In the convergent approach, the branches of external arms are synthesised first as dendrons, and subsequently bonded to a core structure. In the divergent approach the dendrimer is synthesised from the core, through step by step addition of a suitably protected monomer building block. Dendrimer synthesis still remains a hard challenge, due to the large number of reaction steps required; in order to facilitate the access to these promising macromolecules, accelerated approaches have been proposed in the last years.

Dendrimers can be classified in several sub-families, based on their structural features, ranging from monodisperse dendrons and dendrimers, to polydispersed dendrigrafts and dendritic-linear hybrids. In this work, we present the synthesis of a novel hyperbranched monodisperse linear dendrimer, based on 2,2-bis-(hydroxymethyl)-propionic acid (bis-MPA), by convergent metathesis-mediated coupling between the alkene-terminated focal point of bis-MPA dendrons. To the best of our knowledge, this is the first example of the use of metathesis for focal point coupling. Metathesis, instead, has been used in olefin cross-metathesis on polyolefin dendrimer surfaces, for the internal incorporation of guest molecules into a dendrimer, for post-synthetic modification of glycodendrons at focal points.
for the synthesis of cored dendrimers,\textsuperscript{15,16} for dendritic linear hybrids through ring-opening metathesis polymerization,\textsuperscript{17} and for cross-linking of hyperbranched dendrimeric structures.\textsuperscript{18}

**Experimental General**

Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60F\textsubscript{254} coated glass plates (Merck). The spots were visualized by charring with a conc. H\textsubscript{2}SO\textsubscript{4}/EtOH/H\textsubscript{2}O solution (10:45:45 v/v/v), or with a solution of (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24} (21 g), Ce(SO\textsubscript{4})\textsubscript{2} (1 g), conc. H\textsubscript{2}SO\textsubscript{4} (31 mL) in water (500 mL) and then by heating to 110 °C for 5 min. Flash column chromatography was performed on silica gel 230–400 mesh (Merck).

Routine \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian Mercury instrument at 400 MHz (\textsuperscript{1}H) and 100.57 MHz (\textsuperscript{13}C). Chemical shifts are reported in parts per million downfield from TMS as an internal standard; \textit{J} values are given in Hz. Mass spectra were recorded on a QSTAR Elite instrument (AB Sciex) equipped with a nano-electrospray ion source. The samples were directly injected at room temperature by borosilicate capillaries (Thermo Scientific) employing a spray voltage of 1.1 kV and a declustering potential of 80 V.

**Synthesis of 1, 5, 9**

In order to obtain several progressive family of dendrons, sequential condensation reactions were performed following modified literature procedures starting from 2,2,5-Trimethyl-1,3-dioxane-5-carboxylic acid as a building block through sequential condensation steps (DCC, PPT 0.04 eq., DCM dry 0.1M, r.t.),\textsuperscript{19} and deprotection. Products were purified by Flash chromatography column and obtained in 20-40 % yields using a mixture of petroleum spirit-EtOAc as eluent. All reagents were purchased from Sigma-Aldrich and used without any further purification.
**Boc-protected tetravalent dendrimer 2.**

425 mg of compound 1 (0.775 mmol), 22 mg of Hoveyda Grubbs 2nd generation catalyst (4 % mol in respect to 1), were dissolved in anhydrous octafluorotoluene (0.1M). The solution was stirred at room temperature, in the dark and under argon atmosphere. After 48 hr the solvent was evaporated under vacuum and then the crude product was purified by flash chromatography (Petroleum ether/ EtOAc 6:4), affording compound 2 (152 mg, 37 % yield).

$^1$H NMR (400 MHz, CDCl$_3$) \( \delta \) 7.92 (bs, 4H, NH-O), 5.40 (bs, 2H, CH=CH), 4.43 (s, 8H, NH-O-CH$_2$), 4.34 (ABq, \( J = 11.1 \) Hz, 8H, CO-CH$_2$), 4.13 (t, \( J = 6.4 \) Hz, 4H, CO-CH$_2$-CH$_2$), 2.07 – 1.97 (bs, 4H, CH$_2$-CH=), 1.74-1.64 (m, 4H, CH$_2$-CH$_2$-CH), 1.47 (s, 36H, CH$_3$(Boc)), 1.27 (s, 6H, CH$_3$). $^{13}$C NMR (101 MHz, CDCl$_3$) \( \delta \) 172.34 (2s, 2COO), 169.15 (4s, 4COO), 156.22 (4s, 4CONH), 129.52 (2d, 2CH=CH), 82.23 (4s, 4C(Boc)), 72.34 (4t, 4NH-O-CH$_2$), 65.42 (4t, 4COOCH$_2$C), 64.92 (2t, 2COOCH$_2$CH$_2$), 46.30 (2t, 2C(CH$_2$OCO)$_2$), 28.65 (2t, 2CH$_2$-CH=), 28.33 (2t, 2CH$_2$-CH$_2$-CH=), 28.14 (12q, 12CH$_3$(Boc)), 17.93 (2q, 2CH$_3$).

NanoESI-MS: C$_{46}$H$_{76}$O$_{24}$N$_4$ calcd mass 1068.48, observed m/z = 1069.10 (H$^+$ adduct) and m/z = 1091.10 (Na$^+$ adduct), experimental mass 1068.00.

**Deprotected tetravalent dendrimer 3.**

A solution of 2 (108 mg, 0.101 mmol) in anhydrous dichloromethane (DCM, 0.1M) was cooled to 0 °C, then trifluoroacetic acid (TFA) was added dropwise and stirred for 2 hr. The reaction was quenched with satd. aq. Na$_2$CO$_3$ to neutrality, and extracted twice with DCM, the organic layers were collected and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated affording product 3 quantitatively (66 mg). The product was used for the subsequent reaction without any further purification. $^1$H NMR (400 MHz, CDCl$_3$) \( \delta \) 5.81 (bs, 8H, NH$_2$-O), 5.39 (bs, 2H, CH=CH), 4.33 (ABq, \( J = 11.1 \) Hz, 8H, CO-CH$_2$), 4.22 (s, 8H, NH-O-CH$_2$), 4.11 (t, \( J = 6.5 \) Hz, 4H, CO-CH$_2$-CH$_2$), 2.11 – 1.98 (bs, 4H, CH$_2$-CH=), 1.75 –
1.58 (m, 4H, CH₂-CH₂-CH₂-CH₂), 1.24 (s, 6H, CH₃). \(^{13}\)C NMR (101 MHz, CDCl₃) δ 172.46 (2s, 2COO), 170.30 (4s, 4COO), 129.79 (2d, 2CH=CH), 72.27 (4t, 4NH-O-CH₂), 65.45 (4t, 4COOCH₂C), 64.77 (2t, 2COOCH₂CH₂), 46.31 (2s, 2C(CH₂OCO)₂), 28.65 (2t, 2CH₂+CH₂), 28.17 (2t, 2CH₂+CH₂-CH), 17.88 (2q, 2CH₃). NanoESI+MS: C\(_{26}\)H\(_{44}\)O\(_{16}\)N\(_{4}\) calcd mass 668.27, observed m/z = 669.00 (H\(^+\) adduct), experimental mass 668.00.

**Tetravalent glycosylated dendrimer 4**

To obtain multifunctional glycosylated dendrimer 4, 25 mg of compound 3 (0.037 mmol) were dissolved in 2.5 ml acetate buffer (pH 4.5), then maltose (101 mg, 0.296 mmol and aniline (41 mg, 0.444 mmol) were added respectively. After 72hr the product was recovered by washing with a small amount of DCM. The aqueous phase was concentrated to dryness and crude product purified by flash chromatography, affording compound 4 (22 mg, 30 % yield). \(^{1}\)H NMR (400 MHz, D₂O) δ 7.48 (d, \(J = 6.0\) Hz, 3H, CH= N), 7.42 (d, \(J = 6.3\) Hz, 1H, CH=N), 5.31 (bs, 2H, CH=C), 2.11 – 1.98 (bs, 4H, CH₂+CH₂), 1.78 (2q, 2CH₃). NanoESI-MS: C\(_{74}\)H\(_{124}\)O\(_{56}\)N\(_{4}\) calcd mass 1964.70, observed m/z = 1965.35 (2Na\(^+\) adduct), experimental mass 1964.70.

**Boc-protected octavalent dendrimer 6**

A solution of compound 5 (834 mg, 0.740 mmol) and 18 mg Hoveyda Grubbs 2\(^{nd}\) generation catalyst (4 % in mol in respect to 5) was prepared in anhydrous octafluorotoluene (0.2 M), and reacted as described for compound 2. After 24 hr the solvent was evaporated by rotary evaporator, and the crude product was purified by flash chromatography (Petroleum ether/EtOAc 5:5), affording pure product 6 (143 mg, 30 % yield calcd on reacted 5), and 350 mg of unreacted 5. \(^{1}\)H NMR (400 MHz, CDCl₃) δ 8.02 (bs, 8H, NH-O), 5.42 (bs, 2H, CH=CH), 4.44 (s, 16H, NH-O-CH₂), 4.35 – 4.23 (ABq, \(J = 11.2\) Hz, 24H, CO-CH₂), 4.10 (t, \(J = 6.4\) Hz, 4H, CO-CH₂-CH₂), 2.11-2.00 (bs, 4H, CH₂-CH=), 1.69 (bs, 4H, CH₂-CH₂-CH), 1.47 (s, 72H, CH₂(Boc)), 1.24 (s, 18H, CH₃). \(^{13}\)C NMR (101 MHz, CDCl₃) δ 171.67 (2s, 2COO), 169.16
Deprotected octavalent dendrimer 7

A solution of 6 (210 mg, 0.094 mmol) in dry DCM (0.1M) was cooled to 0 °C, then TFA was added dropwise and stirred for 2 hr at 0°C. The reaction was quenched with satd. aq. Na₂CO₃ to neutrality, and the crude product was extracted twice with DCM, and the organic layers dried over anhydrous Na₂SO₄. The solvent was evaporated to give compound 7 (114 mg, 85 % yield), that was used for the subsequent reaction without any further purification. ¹H NMR (400 MHz, CDCl₃) δ 6.22-5.50 (bs, 16H, NH₂-O), 5.42 (bs, 2H, CH=CH), 4.35 – 4.23 (bs, 32H, NH+O+CH₂), 4.17 (8H, CO+CH₂), 4.11 (t, J = 6.5 Hz, 4H, CO+CH₂+CH), 2.14-1.98 (bs, 4H, CH₂-CH), 1.75-1.64 (bs, 4H, CH₂-CH₂-CH), 1.25 (s, 18H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 172.10 (2s, 2COO), 171.84 (4s, 4COO), 170.31 (8s, 8COO), 129.78 (2d, 2CH=CH), 72.25 (8t, 8CO-CH₂), 65.65 (4t, 4CO-CH₂), 65.34(8t, 8NH-O-CH₂), 64.97 (2t, 2 COCH₂CH₂), 46.54, 46.46 (6s, 6C(CH₃)₃), 28.51 (2t, 2t, 2CH₂-CH=), 28.22 (2t, 2CH₂-CH₂-CH=), 17.79 (6q, 6CH₃). NanoESI-MS: C₇₄H₁₅₂O₃₆N₈ calcld mass 1424.53, observed m/z = 713.50 (2H⁺ adduct), experimental mass 1425.00.

Glycosylated dendrimer 8

81 mg of compound 7 (0.057 mmol) were dissolved in 2.5 ml acetate buffer (pH=4.5), subsequently 312 mg of maltose and 126 mg aniline (0.912 mmol and 1.36 mmol
respectively) were added and the mixture allowed to stir. After 72 hr the reaction was stopped and washed twice with DCM. Finally, crude product was purified through flash chromatography (EtOH + 0.2 % AcOH), affording compound 8 (13 mg, 6% yield).

$^1$H NMR (400 MHz, D$_2$O) δ 7.47 (d, $J = 6.1$ Hz, 5H, CH=N), 7.41 (d, $J = 6.6$ Hz, 1H, CH=N), 5.31 (bs, 2H, CH=CH), 2.11 – 1.98 (bs, 4H, CH$_2$-CH). NanoESI+MS: C$_{150}$H$_{248}$O$_{116}$N$_8$ calcd mass 4017.37, observed m/z = 1832.50 (2Na$^+$ adduct, minus one aminoxyacetic moiety, C$_{14}$H$_{24}$O$_2$N), m/z = 1634.00 (2Na$^+$ adduct, minus two aminoxyacetic moieties) and m/z = 1435.50 (2Na$^+$ adduct, minus three aminoxyacetic moieties), experimental mass 4017.13.

**Boc-protected hexadeca dendrimer 10**

Compound 9 (110 mg, 0.048 mmol) and 2 mg (7 % mol in respect to 9) Hoveyda Grubbs 2nd generation dissolved in anhydrous octafluorotoluene (0.2 M), were reacted as described for compound 2. After 72 hr the solvent was evaporated under vacuum and then the crude product was purified by flash chromatography (Petroleum ether/ EtOAc 4:6) to afford pure 10 (34 mg, 31 % yield). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.10 (s, 16H, NH-O), 5.44 (bs, 1H, CH=CH), 5.12 (bs, 1H, CH=CH), 4.45 (s, 32H, NH-O-CH$_2$), 4.31- 4.20 (ABq, $J = 11.6$ Hz, 56H, CO-CH$_2$), 4.11 (t, $J = 6.6$ Hz, 4H, CO-CH$_2$-CH$_2$), 2.02 (bs, 4H, CH$_2$-CH), 1.66 (bs, 4H, CH$_2$-CH$_2$-CH), 1.47 (s, 144H, C(CH$_3$)$_3$(Boc)), 1.26 (bs, 42H, CH$_3$). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 173.54 (2s, 2COO), 171.69 (4s, 4COO), 171.43 (8s, 8COO), 169.22 (16s, 16CONH), 169.02 (16s, 16CONH), 82.11 (16s, 16CONH), 72.34 (16t, 16NH-O-CH$_2$), 65.84, (16t, 16CO-CH$_2$), 65.26 (12t, 12CO-CH$_2$), 65.06 (2t, 2COOCH$_2$CH$_2$) 46.64, 46.43(14s, 14(C(CH$_3$)CO)$_2$), 34.01 (2CH$_2$-CH), 31.83 (2t, 2CH$_2$-CH$_2$-CH), 28.16 (48q, 48CH$_3$(Boc)), 17.79 (14q, 14CH$_3$).

NanoESI-MS: C$_{190}$H$_{304}$O$_{108}$N$_{16}$ calcd mass 4537.88 , observed m/z = 2291.90 (2Na$^+$ adduct), experimental mass 4537.80.
Results and Discussion

Dendritic structures based on bis-MPA are biocompatible, non immunogenic and constituted by a degradable polyester backbone, thus eligible as promising scaffolds for a wide collection of biomedical applications.\textsuperscript{20, 21, 22, 23} Carbohydrates are known to be fundamental biomolecules for cellular signaling; for example, through their interactions with lectins, a family of carbohydrate-binding proteins, they are involved in the immune response, in cellular recognition and adhesion.\textsuperscript{24, 25, 26} In this study we propose a new methodology for the synthesis of multivalent glycodendrimers, using a metathesis reaction with Hoveyda–Grubbs 2\textsuperscript{nd} generation catalyst in order to obtain symmetrical dimers with high branching degree; the terminal ends of the dendrimers expose multiple aminoxyl groups that can be exploited for glycoconjugation of unprotected sugars. These branched structures may provide good benefit to biomedical and tissue engineering applications, where high density of ligand exposure and spatial topographical presentation are crucial to bring about desired biological effects.\textsuperscript{27, 28, 29}

Starting from a bivalent (1), tetravalent (5) (both previously synthesized in our laboratory)\textsuperscript{30, 31} and octavalent (9) dendron monomers with a core double bond (Scheme 1) we achieved the synthesis of symmetrical dendrimers, doubling the branching degree of each structure by a single-step metathesis reaction. Dendrimers 2 and 6 were synthesized from dendrons 1 and 5 via a metathesis reaction with Hoveyda–Grubbs 2\textsuperscript{nd} generation using octafluorotoluene as solvent, because it permits a faster and more efficient reaction by boosting the activity of the catalyst.\textsuperscript{32} Each reaction was followed by visualization on thin-layer chromatography.

Protected dendrimers 2 and 6 were then reacted with trifluoroacetic acid for the removal of \textit{t}-butoxycarbonyl (Boc) protecting groups, leading to deprotected dendrimers 3 and 7. The aminoxyl-branched dendrimers were reacted with maltose, as sample saccharide, in the presence of aniline as catalyst in acetate buffer at pH 4.5, yielding glycodendrimers 4 and 8, exposing multiple sugar moieties at their ends, potentially capable of eliciting a biological
response in a biomedical context. The addition of aniline for the aminoxycarbonyl coupling permits to shift the equilibrium from the stable carbohydrate cyclic hemiacetal form towards a more reactive open-chain intermediate,\(^{33}\) enhancing the reaction with the aminoxy groups. Maltose, once exposed on the dendrimers will present an α-glucoside epitope, which is a fundamental signaling moiety in a variety of biochemical interactions.\(^{34, 35}\) In order to demonstrate the efficacy of the metathesis reaction, the synthesis of the hexadeca-valent dendrimer 10 was performed from dendron 9 with the same procedure described before.

All compounds have been analysed by NMR spectroscopy and MS spectrometry. However, as the complexity of the dendrimeric structure increases, spectra show high degree of overlapping signals. In these cases most significant peaks have been identified. MS analyses confirmed their masses, with the exception of glycodendrimer 8 (see below).

In more details, for glycodendrimers 4 and 8, NMR spectra resulted of difficult interpretation, due to the highly crowded pattern of signals belonging to the sugars, to the bis-MPA skeleton, to possible E/Z isomerism of oxime bonds and to the amphipathic nature of these molecules; in fact, as glycosidation proceeds in water, dendrimers may tend to aggregate into micellar structures masking the hydrophobic backbone. However, regarding tetravalent glycodendrimer 4, \(^1\)H NMR spectra clearly show the oxime and double bond signals with relative integral of ~4:2, as expected from the presence of four sugar moieties and confirmed by MS analysis.

Considering octavalent glycodendrimer 8 \(^1\)H NMR spectra, the ratio between oxime and double bond signals suggests a partial glycosydation, with an integral ratio of about 7:2 (see experimental section). MS analysis of dendrimer 8 however shows the presence of partially degraded products deriving from the loss of one, two or three terminal aminoxycetic groups.
Considering hexadeca dendrimer 10 NMR spectra, they are much more complicated than the others, due to the high complexity of its structure; however, $^1$H NMR spectrum displays the expected double bond signals and a peak corresponding to the doubly sodiated molecule (m/z = 2292) has been detected in the MS spectrum, thus confirming the presence of the product.

**Conclusions**

Cell-cell and cell-environment interaction are mediated by protein-carbohydrate recognition processes at cell surface during the first step of cellular sensing, triggering a wide variety of biological events. Cell-cell and cell-environment interactions are known to be mediated also by carbohydrates. In this context, highly branched glycosylated structures are interesting tools to enhance these recognition events, helping in elucidating the biological role behind carbohydrate as signalling molecules or acting as antagonist of relevant recognition events (i.e. involving viruses or bacteria). Here we have presented a novel method for the synthesis of dendrimers by olefin metathesis of dendron precursors, and explored the glycoconjugation reaction toward glycodendrimers, useful to generate highly branched structures displaying multiple carbohydrate moieties. The obtained structures present an internal double bond which might be further reduced to the corresponding saturated structure, thus allowing an increase in conformational flexibility, enlarging possibilities of fruitful interactions with biological targets. In addition, the length of the hydrocarbon chain can be varied at wish (i.e. alkene-terminated linear polymers can be used as the dendron core), allowing an additional degree of structural variation. Moreover aniline has been used as stabilizer of glycosyl intermediate for improving dendron glycoconjugation. It should be noted, however, that as the complexity of the structure increases, side reactions may occur, affording partially degraded products, as already reported for this kind of hyperbranched structures.36
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(2) Wu, L.; Ficker, M.; Christensen, J. B.; Trohopoulos, P.N. and Moghimi, S. M. Bioconj. Chem. 2015, 26, 1198–1211. DOI: 10.1021/acs.bioconjchem.5b00031.


Figures & Schemes

Scheme 1
Graphical Abstract.

Scheme 1: Reactions scheme. Conditions: a) Hoveyda-Grubbs 2nd, Octafluorotoluene; b) TFA 30%, DCM dry; c) Maltose, Aniline, Acetate Buffer pH 4.5
Olefin metathesis

2 x bis-MPA

= ONH₂