Second generation dendrimers with CS-E disaccharides as multivalent ligands for Langerin.

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ABSTRACT

Chondroitin sulfate type E (CS-E) is a sulfated polysaccharide that shows several interesting biological activities, such as the modulation of the neuronal growth factor signaling and its interaction with Langerin, a C-type lectin with a crucial role in the immunological system. However, applications of CS-E are hampered by the typical heterogeneous structure of the

natural polysaccharide. Well-defined, homogeneous CS-E analogues are highly demanded. Here, we report the synthesis of monodisperse, structurally well-defined second-generation glycodendrimers displaying up to 18 CS-E disaccharide units. These complex multivalent systems have a molecular weight and a number of disaccharide repeating units comparable with those of the natural polysaccharides. In addition, SPR experiments revealed a calcium-independent interaction between these glycodendrimers and Langerin, in the micromolar range, highlighting the utility of these compounds as CS-E mimetics.

Introduction

Chondroitin sulfate (CS) is a polysaccharide that belongs to the glycosaminoglycan (GAG) family, formed by alternating D-glucuronic acid (GlcA) and N-acetyl D-galactosamine (GalNAc) moieties. This disaccharide repeating unit is decorated with sulfate groups at different positions. Several classes of CS can be distinguished depending on the presence of a predominant sulfation motif. Interestingly, particular sulfate distributions are responsible for the specific interaction between CS and certain protein receptors that regulate important biological processes.[1] For instance, CS type E is characterized by disaccharide repeating units containing two sulfate groups at positions 4 and 6 of the GalNAc residues. It has been shown that CS-E as well as heparin are able to bind Langerin, a key C-type lectin of the immunological system, in a calcium-independent manner.[2, 3] The interaction between these GAGs and Langerin is crucial for the biological role of this lectin, mediating the recognition of certain pathogens, such as the HIV virus,[4] their internalization into Birbeck granules and finally their elimination. Therefore, the use of pure CS sequences with a well-defined sulfation pattern is needed for establishing the precise structural requirements of the molecular recognition between Langerin and CS. These studies

will afford valuable data, at the molecular level, on this interaction and will open the way to modulate the mechanism of the organism response against pathogen infections in which Langerin is involved.

However, natural CS chains do not display a homogeneous composition and the major sulfate distribution is usually accompanied by other minor sulfation motifs.[5] This structural heterogeneity significantly hampers the biomedical applications of these carbohydrates, the establishment of structure-activity relationships and the unambiguous study of the molecular recognition processes between CS and proteins. In this context, the development of CS multivalent systems is a very attractive approach for the preparation of polysaccharide mimetics showing a well-defined sulfation pattern. Importantly, multivalent presentation of a CS disaccharide ligand increases its protein binding affinity due to the multivalent effect.[6] In this way, the conjugation of a synthetic CS epitope on an appropriate scaffold, such as a polymer[7, 8], a dendrimer[9-11] or an oligopeptide[12], have given rise to different CS analogues that imitate the structure and biological activities of the natural polysaccharide.

We have previously described the preparation of first-generation glycodendrimers displaying 3, 4 and 6 CS-E disaccharide ligands.[13] However, these multivalent systems presented a limited number of repeating units that was far from the size of the biologically active polysaccharides. To better mimic the biological activities of the natural products, multivalent systems showing a higher valency are required. The chemical synthesis and structural characterization of this type of molecules is very challenging due to the presence of a high number of negative charges (sulfates and carboxylates). Here, we have expanded our synthetic strategy to the preparation of second-generation CS-E glycodendrimers bearing 9, 12 and 18 disaccharides. These complex systems present a number of disaccharide repeating units similar to that displayed by natural CS chains.[14]

The dendrimer scaffolds selected have been previously used in our laboratory to successfully prepare glycodendrimers based on pseudomannosides. These systems were capable to interact efficiently with the receptor DC-SIGN with the aim to inhibit the infection of cells by different virus (Ebola, Zika, Dengue).[15] In addition, SPR experiments have shown the interaction between Langerin and the synthesized second-generation dendrimers. Our results demonstrate the utility of these glycomimetics as powerful tools for the study of the molecular recognition between CS and proteins.

Experimental Section

General synthetic procedures: Thin layer chromatography (TLC) analyses were performed on silica gel 60 F_{254} precoated on aluminium plates (Merck) and the compounds were detected by staining with cerium (IV) sulfate (1 g)/ ammonium molybdate tetrahydrate (21 g)/sulfuric acid (30 mL) solution in water (0.47 L), or with anisaldehyde solution [anisaldehyde (25 mL), sulfuric acid (25 mL) and acetic acid (1 mL) in ethanol (450 mL)], followed by heating at over 200ºC. Size exclusion chromatography was performed using Sephadex LH-20 gel from GE Healthcare. Ion-exchange chromatography was carried out using Dowex 50WX2 Na^+ resin from Sigma-Aldrich. For ultrafiltration, Amicon Ultra-4 devices from Merck were employed. ^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker Avance III-400 spectrometer. Bidimensional COSY and HSQC NMR experiments were carried out to assist in signal assignment. Electrospray mass spectra (ESI MS) of dendrons **3** and **4** were carried out with an Esquire 6000 ESI-Ion Trap from Bruker Daltonics, using a 10 mM diethylamine solution in a 1:1 MeOH/H_2O mixture. To weigh compounds, such as the dendritic cores, in quantities lower than 1 mg, we prepared a stock solution and took the appropriate aliquot from it. After concentration and drying under high vacuum, we checked the weight by using a semi microbalance (5 decimal places).

Dendrimer size and zeta potential measurements: The dendrimer diameter was determined by dynamic light scattering (DLS) using a Malvern Nano ZS (Malvern Panalytical, U.K.) instrument equipped with a 633 nm laser. Compounds **6**, **8** and **10** were dissolved at a 150 μM concentration in 25 mM Tris buffer (pH 8, 150 mM NaCl) both in the presence and absence of 4 mM $CaCl_2$. All the solutions were passed through a 0.22 μm PVDF (Polyvinylidene Fluoride) filter (Millipore) before the DLS measurements. Samples were recorded at a 173º scattering angle and data were analyzed using the number distribution mode. The reported mean diameter values resulted from at least 6 measurements.

The zeta potential was also measured using a Malvern Nano ZS equipment. In this case, glycodendrimers were dissolved in MilliQ water. At least 3 measurements per sample were recorded and the mean and standard deviation from these measurements were reported.

MALDI-ToF experiments: MALDI-ToF mass spectra of glycodendrimers **6**, **8** and **10** were acquired with a Microflex LRF spectrometer from Bruker fitted with a 337 nm nitrogen laser. A saturated sinapinic acid solution in water/acetonitrile 70:30 (v/v) with 0.1% trifluoroacetic acid was used as matrix. Recombinant human FGF-2 was purchased from Peprotech. $(Arg\text{-}Gly)_{15}$ peptide was synthesized by the Peptides Synthesis Service, Facultad de Química, Barcelona. For glycodendrimers **6** and **8**, 2 μL of a 10 μM FGF-2 solution containing 0.05% v/v trifluoroacetic acid were mixed with 2 μL of a 10 μM glycodendrimer solution (also containing 0.05% v/v trifluoroacetic acid) and with 2 μL of the matrix solution. In the case of glycodendrimer **10**, 2 μL of a 20 μM $(Arg\text{-}Gly)_{15}$ solution containing 0.05% v/v trifluoroacetic acid were mixed with 2 μL of a 20 μM **10** solution (also containing 0.05% v/v trifluoroacetic acid) and with 2 μL of the matrix solution. The MALDI-ToF mass spectra of the resulting mixtures were acquired in the positive ion mode.

Dendron 3: A solution of $CuSO_4 \cdot 5H_2O$ (17 mg, 0.07 mmol) and TBTA (69.7 mg, 0.13 mmol) in DMSO/10 mM PBS buffer 7:1 (3.25 mL) and a solution of sodium ascorbate (38.1 mg, 0.19 mmol) in PBS (0.4 mL, 10 mM) were added to a solution of **1**[13] (97 mg, 0.14 mmol) and **2**[15-17] (10.9 mg, 0.03 mmol) in DMSO/10 mM PBS buffer 8:5 (3.15 mL). The solution was stirred at room temperature overnight. Then, Cu was removed using Quadrasil MP resin and the reaction mixture was purified by size exclusion Sephadex LH-20 chromatography using H_2O/MeOH 9:1 as eluent and ion exchange DOWEX Na^+ chromatography (H_2O/MeOH 9:1) to afford **3** as a sodium salt (57 mg, 75%); ^{1}H-NMR (400 MHz, D_2O): δ 7.92 (s, 3H, Htriazol), 4.79 (s, 3H, H-4), 4.58-4.35 (m, 18H, H-1, H-1', Triazol-CH_2-O, O-CH_2-CH_2-C*H*$_2$-Triazol), 4.39-4.10 (m, 6H, H-6a, H-6b), 4.09-3.96 (m, 9H, H-2, H-3, H-5), 3.92-3.79 (m, 3H, O-C*H*$_2$-CH_2-CH_2-Triazol), 3.74-3.63 (m, 5H, H-5', DEG [O-C*H*$_2$-CH_2-Cl]), 3.63-3.57 (m, 2H, DEG [-C*H*$_2$-Cl]), 3.57-3.42 (m, 13H, H-3', H-4', O-C*H*$_2$-CH_2-CH_2-Triazol, O-C*H*$_2$-C*H*$_2$-O), 3.42-3.27 (m, 11H, H-2', CH_2 pentaerythritol core), 2.11 (m, 6H, O-CH_2-C*H*$_2$-CH_2-Triazol), 1.99 (s, 9H, NHAc); ^{13}C-NMR (100 MHz, D_2O): δ 175.3, 174.7 (2 x CO), 144.1 (Ctriazol), 125.0 (CHtriazol), 103.4 (C-1'), 101.2 (C-1), 76.2 (C-4), 76.0 (C-5'), 75.1 (C-3, C-3'), 72.5 (C-2'), 72.3 (C-5), 71.7 (C-4'), 70.8 (DEG [O-*C*H_2-CH_2-Cl]), 70.3 (O-CH_2-*C*H_2-O or O-*C*H_2-CH_2-O), 69.5 (O-*C*H_2-CH_2-O or O-CH_2-*C*H_2-O), 68.3 (CH_2 pentaerythritol core), 67.9 (C-6), 66.7 (O-*C*H_2-CH_2-CH_2-Triazol), 63.5 (Triazol-CH_2-O), 51.7 (C-2), 47.0 (O-CH_2-CH_2-*C*H_2-Triazol), 44.6 (C pentaerythritol core), 43.4 (DEG [-*C*H_2-Cl]), 29.5 (O-CH_2-*C*H_2-CH_2-Triazol), 22.4 (NHAc); ESI MS: *m/z* calcd for $C_{69}H_{100}ClN_{12}Na_9O_{59}S_6$: 2474.2; found: 1174.3 $[M-9Na+6H+Et_2NH_2]^{2-}$.

Dendron 4: NaN_3 (50 mg, 0.77 mmol) was added to a solution of **3** (42.9 mg, 0.018 mmol) in DMSO (4 mL). The solution was stirred at 60^{o}C for 4 days. Then, the reaction mixture was purified by size exclusion Sephadex LH-20 chromatography using H_2O/MeOH 9:1 as eluent and

ion exchange DOWEX Na^+ chromatography (H_2O/MeOH 9:1) to afford **4** as a sodium salt (39.9 mg, 93%); ^{1}H-NMR (400 MHz, D_2O): δ 7.91 (s, 3H, Htriazol), 4.78 (s, 3H, H-4), 4.56-4.34 (m, 18H, H-1, H-1', Triazol-CH_2-O, O-CH_2-CH_2-C*H*$_2$-Triazol), 4.28-4.09 (m, 6H, H-6a, H-6b), 4.08-3.95 (m, 9H, H-2, H-3, H-5), 3.90-3.77 (m, 3H, O-C*H*$_2$-CH_2-CH_2-Triazol), 3.70-3.61 (m, 3H, H-5'), 3.58 (m, 2H, DEG [O-C*H*$_2$-CH_2-N_3]), 3.55-3.25 (m, 26H, H-2', H-3', H-4', DEG [-C*H*$_2$-N_3], O-C*H*$_2$-CH_2-CH_2-Triazol, O-C*H*$_2$-C*H*$_2$-O, CH_2 pentaerythritol core), 2.10 (m, 6H, O-CH_2-C*H*$_2$-CH_2-Triazol), 1.98 (s, 9H, NHAc); ^{13}C-NMR (100 MHz, D_2O): δ 175.5, 174.7 (2 x CO), 125.0 (CHtriazol), 103.4 (C-1'), 101.2 (C-1), 76.2 (C-4), 76.1 (C-5'), 75.1 (C-3'), 75.0 (C-3), 72.5 (C-2'), 72.3 (C-5), 71.8 (C-4'), 70.4 (O-CH_2-*C*H_2-O or O-*C*H_2-CH_2-O), 69.6 (O-*C*H_2-CH_2-O or O-CH_2-*C*H_2-O), 69.3 (DEG [O-*C*H_2-CH_2-N_3]), 68.3 (CH_2 pentaerythritol core), 67.9 (C-6), 66.8 (O-*C*H_2-CH_2-CH_2-Triazol), 63.5 (Triazol-CH_2-O), 51.7 (C-2), 50.2 (DEG [-*C*H_2-N_3]), 47.0 (O-CH_2-CH_2-*C*H_2-Triazol), 44.6 (C pentaerythritol core), 29.5 (O-CH_2-*C*H_2-CH_2-Triazol), 22.4 (NHAc); ESI MS: *m/z* calcd for $C_{69}H_{100}N_{15}Na_9O_{59}S_6$: 2481.3; found: 1177.3 [M-9Na+6H+Et_2NH_2]$^{2-}$.

Dendrimer 6: A solution of $CuSO_4 \cdot 5H_2O$ (2.2 mg, 0.009 mmol) and TBTA (9.4 mg, 0.017 mmol) in DMSO/10 mM PBS buffer 3:1 (0.5 mL) and a solution of sodium ascorbate (5.2 mg, 0.026 mmol) in PBS (0.12 mL, 10 mM) were added to a solution of **4** (20.0 mg, 0.008 mmol) and **5**[15] (0.5 mg, 1.8 x 10^{-3} mmol) in DMSO/10 mM PBS buffer 3:1 (0.5 mL). The solution was stirred at room temperature overnight. Then, Cu was removed using Quadrasil MP resin and the reaction mixture was purified by size exclusion Sephadex LH-20 chromatography using H_2O/MeOH 9:1 as eluent, ion exchange DOWEX Na^+ chromatography (H_2O/MeOH 9:1) and finally by ultracentrifugation using Amicon Ultra-4 (3 kDa) filters to afford **6** as a sodium salt (11.3 mg, 78%); ^{1}H-NMR (400 MHz, D_2O): δ 8.06 (s, 3H, Htriazol), 7.84 (s, 9H, Htriazol), 6.26

(s, 3H, H aromatic core), 5.07 (s, 6H, Triazol-CH_2-O-Benzene), 4.77 (s, 9H, H-4), 4.54 (s, 6H, DEG [-CH_2-Triazol]), 4.50-4.27 (m, 54H, H-1, H-1', Triazol-CH_2-O- pentaerythritol, O-CH_2-CH_2-C*H*$_2$-Triazol), 4.26-4.07 (m, 18H, H-6a, H-6b), 4.06-3.93 (m, 27H, H-2, H-3, H-5), 3.91-3.73 (m, 15H, O-C*H*$_2$-CH_2-CH_2-Triazol, DEG [O-C*H*$_2$-CH_2-Triazol]), 3.65 (d, 9H, $J_{4,5}$ = 9.1 Hz, H-5'), 3.55-3.36 (m, 39H, H-3', H-4', O-C*H*$_2$-CH_2-CH_2-Triazol, O-C*H*$_2$-C*H*$_2$-O), 3.36-3.19 (m, 33H, H-2', CH_2 pentaerythritol core), 2.04 (m, 18H, O-CH_2-C*H*$_2$-CH_2-Triazol), 1.95 (s, 27H, NHAc); ^{13}C-NMR (100 MHz, D_2O): δ 175.9, 174.7 (2 x CO), 159.6 (C aromatic core-O), 103.4 (C-1'), 101.2 (C-1), 76.3 (C-4, C-5'), 75.1 (C-3', C-3), 72.5 (C-2'), 72.3 (C-5), 71.8 (C-4'), 70.4 (O-CH_2-*C*H_2-O or O-*C*H_2-CH_2-O), 69.6 (O-*C*H_2-CH_2-O or O-CH_2-*C*H_2-O), 68.8 (DEG [O-*C*H_2-CH_2-Triazol]), 68.3 (CH_2 pentaerythritol core), 67.9 (C-6), 66.8 (O-*C*H_2-CH_2-CH_2-Triazol), 63.5(Triazol-CH_2-O-pentaerythritol), 61.3 (Triazol-CH_2-O-benzene), 51.7 (C-2), 50.2 (DEG [-*C*H_2-Triazol]), 47.0 (O-CH_2-CH_2-*C*H_2-Triazol), 44.7 (C pentaerythritol core), 29.5 (O-CH_2-*C*H_2-CH_2-Triazol), 22.4 (NHAc); MALDI-ToF MS: *m/z* calcd for $C_{222}H_{312}N_{45}Na_{27}O_{180}S_{18}$: 7.7 kDa; found: 24.1 kDa [M-27Na+(FGF-2)]$^+$.

Dendrimer 8: A solution of $CuSO_4 \cdot 5H_2O$ (2.5 mg, 0.01 mmol) and TBTA (10.0 mg, 0.018 mmol) in DMSO/10 mM PBS buffer 3:1 (0.5 mL) and a solution of sodium ascorbate (5.4 mg, 0.027 mmol) in PBS (0.12 mL, 10 mM) were added to a solution of **4** (20.8 mg, 8.4 x 10^{-3} mmol) and **7**[15] (0.4 mg, 1.4 x 10^{-3} mmol) in DMSO/10 mM PBS buffer 3:1 (0.5 mL). The solution was stirred at room temperature overnight. Then, Cu was removed using Quadrasil MP resin and the reaction mixture was purified by size exclusion Sephadex LH-20 chromatography using H_2O/MeOH 9:1 as eluent, ion exchange DOWEX Na^+ chromatography (H_2O/MeOH 9:1) and finally by ultracentrifugation using Amicon Ultra-4 (3 kDa) filters to afford **8** as a sodium salt (11.2 mg, 79%); ^{1}H-NMR (400 MHz, D_2O): δ 7.90 (s, 4H, Htriazol), 7.86 (s, 12H, Htriazol),

4.77 (s, 12H, H-4), 4.55-4.29 (m, 88H, H-1, H-1', Triazol-CH_2-O- pentaerythritol, O-CH_2-CH_2-CH_2-Triazol, DEG [CH_2-Triazol]), 4.28-4.09 (m, 24H, H-6a, H-6b), 4.07-3.94 (m, 36H, H-2, H-3, H-5), 3.89-3.77 (m, 20H, O-CH_2-CH_2-CH_2-Triazol, DEG [O-CH_2-CH_2-Triazol), 3.70-3.59 (m, 12H, H-5'), 3.55-3.38 (m, 52H, H-3', H-4', O-CH_2-CH_2-CH_2-Triazol, O-CH_2-CH_2-O), 3.38-3.21 (m, 52H, H-2', CH_2 pentaerythritol core), 2.06 (m, 24H, O-CH_2-CH_2-CH_2-Triazol), 1.96 (s, 36H, NHAc); ^{13}C-NMR (100 MHz, D_2O): δ 175.6, 174.7 (2 x CO), 144.1 (C triazol), 124.9 (CHtriazol), 103.4 (C-1'), 101.1 (C-1), 76.3 (C-4, C-5'), 75.1 (C-3', C-3), 72.5 (C-2'), 72.3 (C-5), 72.0 (C-4'), 70.4 (O-CH_2-CH_2-O or O-CH_2-CH_2-O), 69.5 (O-CH_2-CH_2-O or O-CH_2-CH_2-O), 68.8 (CH_2 pentaerythritol core, DEG [O-CH_2-CH_2-Triazol]) 68.2 (CH_2 pentaerythritol core), 67.8 (C-6), 66.8 (O-CH_2-CH_2-CH_2-Triazol), 63.5 (Triazol-CH_2-O), 51.7 (C-2), 49.9 (DEG [-CH_2-Triazol]), 47.0 (O-CH_2-CH_2-CH_2-Triazol), 44.7 (C pentaerythritol core), 29.5 (O-CH_2-CH_2-CH_2-Triazol), 22.4 (NHAc); MALDI-ToF MS: *m/z* calcd for $C_{293}H_{420}N_{60}Na_{36}O_{240}S_{24}$: 10.2 kDa; found: 26.4 kDa [M-36Na+(FGF-2)]$^+$.

Dendrimer 10: A solution of $CuSO_4 \cdot 5H_2O$ (5.2 mg, 0.02 mmol) and TBTA (21.4 mg, 0.04 mmol) in DMSO/10 mM PBS buffer 3:1 (1 mL) and a solution of sodium ascorbate (11.8 mg, 0.058 mmol) in PBS (0.25 mL, 10 mM) were added to a solution of **4** (20.0 mg, 0.008 mmol) and **9**[15] (0.5 mg, 1.0 x 10^{-3} mmol) in DMSO/10 mM PBS buffer 3:1 (0.4 mL). The solution was stirred at room temperature for 36 h. Then, Cu was removed using Quadrasil MP resin and the reaction mixture was purified by size exclusion Sephadex LH-20 chromatography using H_2O/MeOH 9:1 as eluent, ion exchange DOWEX Na^+ chromatography (H_2O/MeOH 9:1) and finally by ultracentrifugation using Amicon Ultra-4 (10 kDa) filters to afford **10** as a sodium salt (14.1 mg, 88%); ^{1}H-NMR (400 MHz, D_2O): δ 7.91 (s, 6H, Htriazol), 7.86 (s, 18H, Htriazol), 4.78 (s, 18H, H-4), 4.55-4.39 (m, 96H, H-1, H-1', Triazol-CH_2-O- pentaerythritol, DEG [-CH_2-

Triazol]), 4.39-4.29 (m, 36H, O-CH_2-CH_2-CH_2-Triazol), 4.28-4.08 (m, 36H, H-6a, H-6b), 4.07-3.94 (m, 54H, H-2, H-3, H-5), 3.90-3.76 (m, 30H, O-CH_2-CH_2-CH_2-Triazol, DEG [O-CH_2-CH_2-Triazol), 3.72-3.62 (m, 18H, H-5'), 3.56-3.39 (m, 78H, H-3', H-4', O-CH_2-CH_2-CH_2-Triazol, O-CH_2-CH_2-O), 3.39-3.23 (m, 82H, H-2', CH_2 pentaerythritol core), 2.05 (m, 36H, O-CH_2-CH_2-CH_2-Triazol), 1.96 (s, 54H, NHAc); ^{13}C-NMR (100 MHz, D_2O): δ 174.7 (CO), 144.1 (C triazol), 124.8 (CHtriazol), 103.5 (C-1'), 101.1 (C-1), 76.3 (C-4), 75.9 (C-5'), 75.3 (C-3), 75.0 (C-3'), 72.5 (C-2'), 72.2 (C-5), 71.7 (C-4'), 70.4 (O-CH_2-*C*H_2-O or O-*C*H_2-CH_2-O), 69.5 (O-*C*H_2-CH_2-O or O-CH_2-*C*H_2-O), 68.8 (CH_2 pentaerythritol core, DEG [O-*C*H_2-CH_2-Triazol]) 68.2 (CH_2 pentaerythritol core), 67.8 (C-6), 66.8 (O-*C*H_2-CH_2-CH_2-Triazol), 63.5 (Triazol-CH_2-O), 51.6 (C-2), 49.8 (DEG [CH_2-Triazol]), 47.0 (O-CH_2-CH_2-*C*H_2-Triazol), 44.7 (C pentaerythritol core), 29.6 (O-CH_2-*C*H_2-CH_2-Triazol), 22.4 (NHAc); MALDI-ToF MS: *m/z* calcd for $C_{442}H_{634}N_{90}Na_{54}O_{361}S_{36}$: 15.4 kDa; found: 10.2 kDa $[M\text{-}54Na+2(Arg\text{-}Gly)_{15}]^{2+}$.

Surface plasmon resonance analysis.

The extracellular domain (ECD) of Langerin (residues 68-328) was overexpressed and purified as previously described.[18] All experiments were performed on a BIAcore T200 using Series S Sensor Chip CM3. The binding affinities of the different dendrimers for Langerin were appreciated *via* competition experiments against a heparin surface. Flow cell one (Fc1) was functionalized with Strep-Tactin® (IBA Lifesciences) and used as a reference, Fc2 was sequentially functionalized with Strep-Tactin® and 6 kDa biotinylated heparin at 5 µL/min using HBS-P buffer from the manufacturer. The two flow cells were activated by 80 µL of a 0.2 M EDC/0.05 M NHS mixture and functionalized with 170 µg/mL Strep-Tactin®, in 10 mM sodium acetate pH 4. The remaining activated groups were blocked with 80 µL of 1 M ethanolamine. After blocking, the two Fcs were treated at 100 µL/min with 100 µL of 10 mM HCl to remove

non-specifically bound protein and 100 µL of 50 mM NaOH/ 1M NaCl. Finally, 3004 and 2992 RU of StrepTactin were immobilized on Fc1 and Fc2, respectively. 6 kDa biotinylated heparin was immobilized on Fc2 at 5 µg/mL in HBS-P buffer + 0.15M NaCl (0.3M NaCl final concentration to reduce non-specific binding). Non-specific binding was removed by injection of 2 times 10 µL of 2 M NaCl. Finally, 25.5 RU was attached to the Fc2 surface. Binding assays were performed at 10 µL/min, at 20°C, in 25 mM Tris pH 8, 150 mM NaCl, 4 mM $CaCl_2$, 0.05% tween or 25 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 0.05% tween. Regeneration was performed by injection of 350 mM $MgCl_2$ for 5 min.

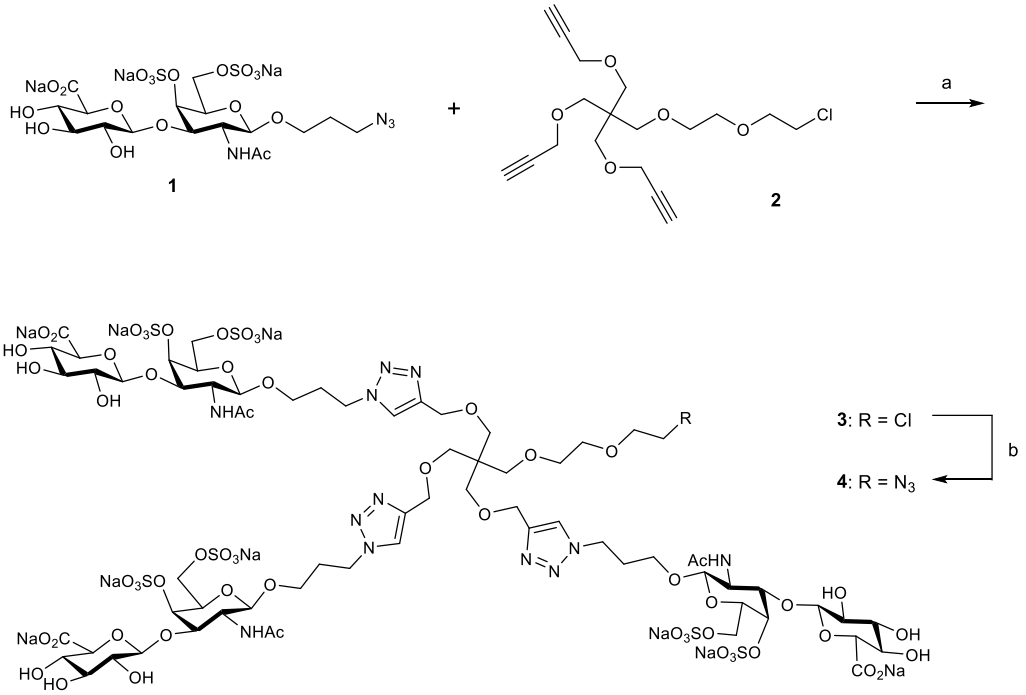
Langerin ECD (20 µM) was injected at 5 µL/min, mixed with a dendrimer concentration range (from 0 to 1.25, 0.2, 1, 0.35, 0.9, 0.5 and 0.4 mM for **11**, **12**, **13**, **14**, **6**, **8** and **10**, respectively). The data were analyzed in BIAcore BIAevaluation software using four parameter equation, and the IC_{50}, defined as compound concentration required for 50% inhibition of the interaction between Langerin and the heparin surface, were determined.

Results and discussion

Synthesis of second-generation glycodendrimers

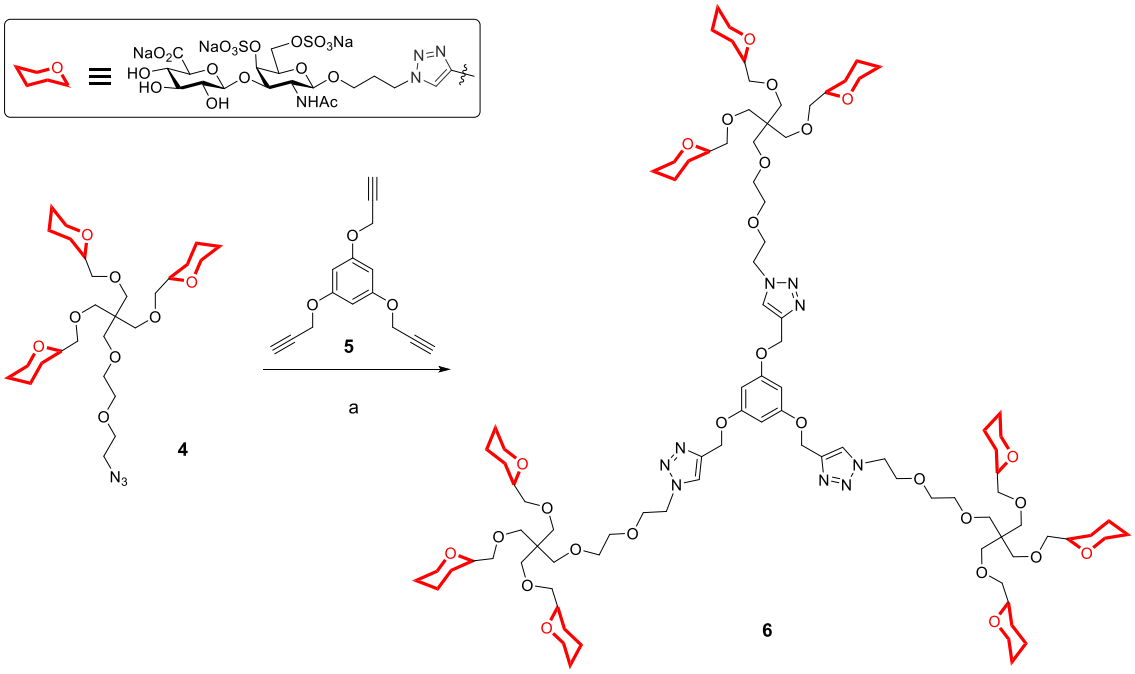
Our synthetic strategy for the preparation of second-generation CS-E dendrimers was based on the use of trivalent glycodendron **4**, conveniently functionalized with an azido group at the focal position for further conjugation (Scheme 1). First, we undertook the synthesis of glycodendron **3** by Cu (I) catalyzed cycloaddition reaction between azide-terminated disaccharide **1** and alkyne-functionalized dendron **2**. Disaccharide **1** showing the typical repeating unit of CS-E was synthesized as previously described using D-glucurono-6,3-lactone and D-galactosamine hydrochloride as starting materials.[13] On the other hand, starting from commercially available

pentaerythritol, dendron **2** was obtained as described in the literature, by introduction of three alkyne groups on the core followed by reaction of the remaining hydroxyl group with 2-chloroethyl ether.[15-17] Conjugation between sugar **1** and the trivalent scaffold **2** was performed in the presence of $CuSO_4$, sodium ascorbate and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA). After purification by size exclusion chromatography and treatment with Quadrasil Mercaptopropyl (MP) to remove the Cu catalyst and Dowex Na^+ resins, glycodendron **3** was obtained in good yield. The ^1H NMR spectrum showed the characteristic signal for the triazole protons at δ = 7.92 ppm. Next, substitution of the chloride by an azido group at the focal position was performed by treatment with sodium azide at 60ºC in DMSO. The structure of compound **4** was confirmed by mass spectrometry and NMR analysis. HSQC NMR experiment was particularly useful and showed the expected ^1H and ^{13}C chemical shifts for the CH_2N_3 group (at δ = 3.33 and 50.2 ppm, respectively) and the disappearance of the signal corresponding to the CH_2Cl moiety of the starting material.



Scheme 1. Synthesis of trivalent glycodendron **4**. Reagents and conditions: a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, DMSO/PBS buffer, room temperature, overnight, 75%; b) NaN_3, DMSO, 60ºC, 4 days, 93%. PBS = phosphate-buffered saline.

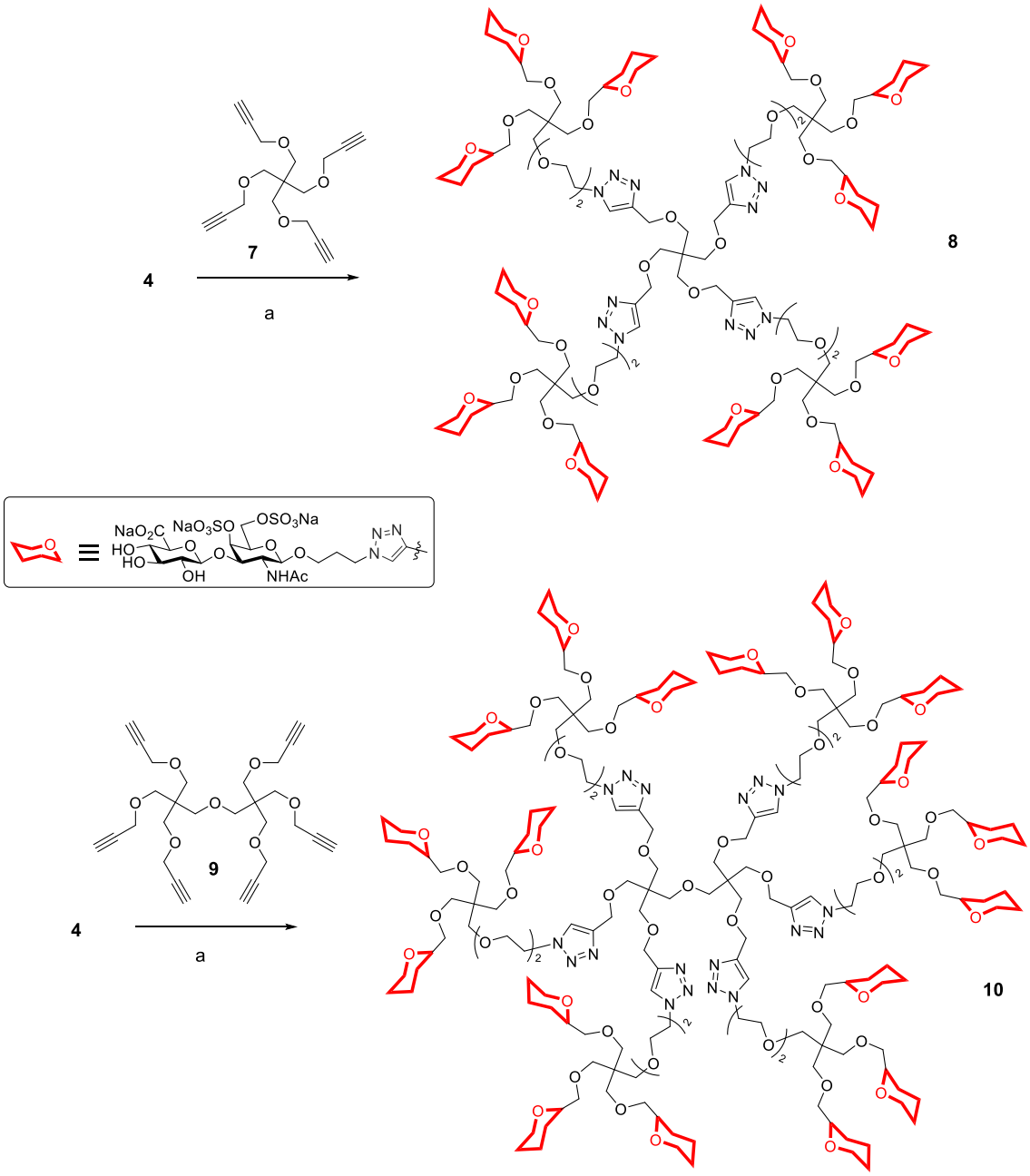
Once the trivalent glycodendron **4** was prepared, we accomplished the preparation of the second generation CS-E dendrimers by conjugation between **4** and a series of alkyne-terminated dendritic cores. Thus, we first performed the Cu (I) catalyzed cycloaddition reaction between **4** and trivalent core **5**, prepared as previously described in the literature from commercially available 1,3,5 tri-hydroxybenzene (Scheme 2).[15] High amounts of $CuSO_4$, TBTA and sodium ascorbate (1.5, 3 and 4.5 equivalents per alkyne group, respectively) were used in order to complete the cycloaddition reaction, preventing the sequestering of the catalyst by the multiple negative charges and hydroxyl groups on the sugar moieties. After a first purification by size exclusion chromatography using Sephadex LH-20 gel, the ^{1}H NMR spectrum still revealed the presence of the starting material **4** along with the desired nonavalent glycodendrimer **6**. Thus, three characteristic peaks for triazole protons were observed: two of them corresponding to glycodendrimer **6** and the third one corresponding to the starting material dendron **4**. Further purification by ultracentrifugation using Amicon Ultra-4 filters (cut-off 3kDa) was required to completely remove the excess of glycodendron **4** (with a molecular weight of around 2.5 kDa) and to obtain pure glycodendrimer **6** in good yield. ^{1}H NMR spectrum showed two singlets at δ = 8.06 and 7.84 ppm in a 1:3 ratio, corresponding to the two types of triazole protons present in dendrimer **6**. Bidimensional COSY and HSQC experiments were carried out for the complete assignment of NMR spectra (see Experimental Part and Supporting Information).



Scheme 2. Synthesis of nonavalent glycodendrimer **6**. Reagents and conditions: a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, DMSO/PBS buffer, room temperature, overnight, 78%.

Using the same strategy, we then synthesized the glycodendrimer **8** bearing 12 disaccharide copies (Scheme 3). For this purpose, glycodendron **4** was conjugated with the known tetravalent core **7**[15] in the presence of an excess of $CuSO_4$, TBTA and sodium ascorbate. The excess of copper salt was removed using Quadrasil MP resin and then the crude mixture was purified by employing Sephadex LH-20 gel, Amicon Ultra-4 filters and Dowex Na^+ resin to afford the glycodendrimer **8** in 79% yield as sodium salt. ^{1}H NMR spectrum displayed the two expected triazole signals (δ = 7.90 and 7.86 ppm) in a 1:4 ratio. Finally, we performed the cycloaddition reaction between **4** and hexavalent dendritic core **9**, prepared as previously described from bis-pentaerythritol,[15] to obtain the glycodendrimer **10** with valence 18 (Scheme 3). In this case, ultrafiltration Amicon membranes with a 10 kDa cut-off were employed to separate compound **10** from the remaining glycodendron **4**. Thus, glycodendrimer **10** was obtained in good yield and

fully characterized by NMR. The detailed assignment of the NMR spectra and the analysis of the proton signals integration indicated the completion of the cycloaddition reaction and the homogeneity of compound **10**.



Scheme 3. Synthesis of glycodendrimers **8** and **10**. Reagents and conditions: a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, DMSO/PBS buffer, room temperature, 79% (**8**), 88% (**10**).

The size of glycodendrimers **6**, **8** and **10** was measured using a dynamic light scattering (DLS) instrument. The glycodendrimers were dissolved at a 150 μM concentration in 25 mM Tris buffer (pH 8, 150 mM NaCl). No aggregates were found and the hydrodynamic diameters could be determined (Table 1 and Supporting Information). Importantly, the addition of calcium ions (4 mM $CaCl_2$) did not induce the formation of aggregates. Thus, the average diameter was from 3.3 nm (for **6**) to 5.2 nm (for **10**) and these values did not change in the presence of 4 mM $CaCl_2$. In addition, the zeta potential of the different glycodendrimers was also determined (Table 1).

	6	**8**	**10**
Diameter (nm)	3.3 ± 0.5	3.6 ± 0.2	5.2 ± 0.2
Zeta potential (mV)	-4.6 ± 0.9	-7.7 ± 0.2	-8.0 ± 1.3

Table 1. Hydrodynamic diameter and zeta potential values obtained for glycodendrimers **6**, **8** and **10**. The reported values are the mean and the standard deviation of at least three measurements.

MALDI-ToF mass spectrometry characterization

The characterization of GAG derivatives by mass spectrometry is a challenging task due to the molecular weight and especially the polyanionic nature of these compounds.[19, 20] Using electrospray ionization (ESI), mass spectra of GAG-like compounds can be difficult to analyze due to the appearance of multiple charged ions and peaks associated with the loss of sulfate groups. While our first-generation CS-E glycodendrimers could be characterized by ESI, the mass spectrometry analysis of the second-generation multivalent systems, with a higher number of negative charges, required a different methodology. MALDI-ToF mass spectrometry is not

employed for the direct characterization of GAGs because sulfated sugars show poor ionization efficiency and no significant signals are usually detected. However, sulfated carbohydrates can be complexed with basic peptides or GAG-binding proteins prior to analysis. The obtained ionic complexes can then be detected by MALDI and the molecular weight of the GAG species can be easily deduced by subtracting the mass of the basic peptide or protein from that of the complex.[21-23]

We chose basic Fibroblast Growth Factor (FGF-2) as protein to form the complex with our CS-E dendrimers **6**, **8** and **10**. It is known that FGF-2 recognizes CS-E sequences[24, 25] and therefore we expected the formation of adducts between FGF-2 and CS-E mimetics **6**, **8** and **10** for MALDI-ToF detection. First, we registered the mass spectrum of recombinant human FGF-2 (see Supporting Information). Peaks corresponding to monomeric (at *m/z* 8554 and 17109) and dimeric FGF-2 (at *m/z* 34133) were detected in good agreement with the theoretical molecular mass of the protein (17.1 kDa). Then, we accomplished the analysis of the complex formed by FGF-2 and nonavalent dendrimer **6**. A saturated sinapinic acid solution in water/acetonitrile 70:30 (v/v) with 0.1% trifluoroacetic acid was used as matrix. 10 μM aqueous solutions of FGF-2 and dendrimer, both containing 0.05% v/v trifluoroacetic acid, were mixed with the matrix solution in a 1:1:1 ratio. The MALDI spectrum of the resulting mixture is shown in Figure 1. We detected peaks corresponding to the protonated 1:1 and 2:1 complexes between FGF-2 and dendrimer **6**. After subtracting the molecular weight of the protein, we deduced a molecular mass of 7.1 kDa for compound **6**, in good agreement with the theoretical value (7.1 kDa). A similar result was obtained with dendrimer **8** (Figure 1). Complexes with 1:1 and 2:1 stoichiometry were detected and a molecular weight of 9.3 kDa was calculated from these data for derivative **8** (9.4 kDa).

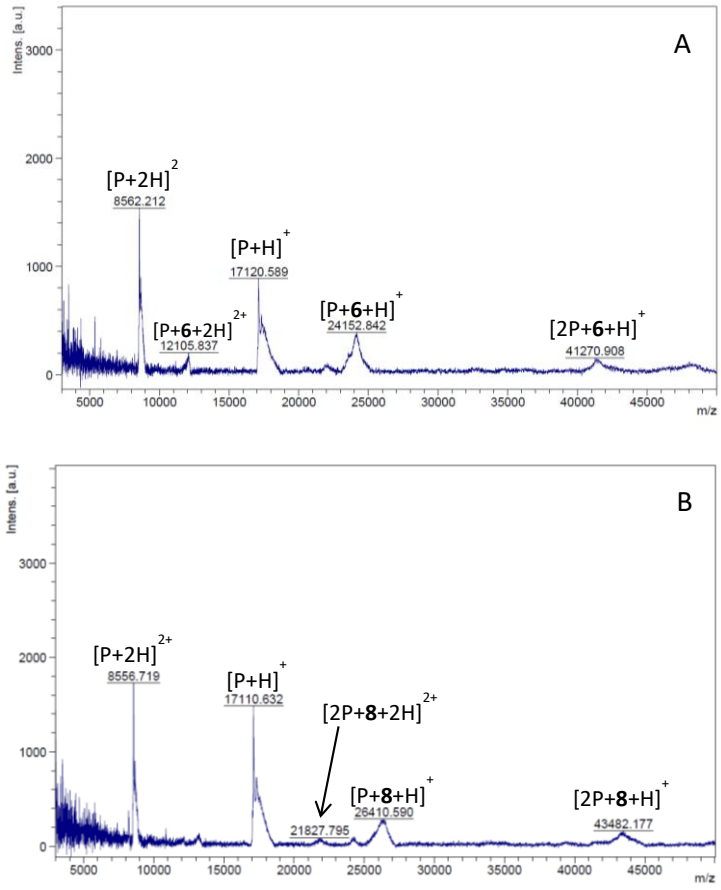


Figure 1. MALDI-ToF mass spectra of equimolecular mixtures of FGF-2 and dendrimers **6** (A) and **8** (B). The letter "P" stands for the protein (FGF-2).

Finally, we attempted the MALDI analysis of dendrimer **10** complexed with FGF-2. However, in this case we could not observe the signals corresponding to the expected ionic complexes. Additional experiments were carried out changing the ratio of FGF-2 to dendrimer **10** (from 1:10 to 10:1) but no complex was detected.[22] For this reason, we decided to employ a synthetic arginine-rich peptide, $(Arg\text{-}Gly)_{15}$ previously used in our group,[26] in order to test the formation of the ionic non-covalent complex with compound **10**. As shown in Figure 2, the most abundant peak (at *m/z* 10245) corresponded to the doubly protonated 2:1 peptide:dendrimer complex. In this case, no 1:1 complex was detected because two copies of the $(Arg\text{-}Gly)_{15}$ peptide were

needed to neutralize the negative charges of dendrimer **10**. Considering that the molecular weight of the $(Arg\text{-}Gly)_{15}$ peptide was 3215 Da, we could deduce a molecular mass of 14.1 kDa for dendrimer **10** in good accordance with the theoretical value (14.2 kDa). This data confirmed the homogeneity of this glycodendrimer containing 18 copies of the disaccharide CS-E ligand. To the best of our knowledge, derivative **10** is the monodisperse GAG multivalent system with highest valence reported to date.

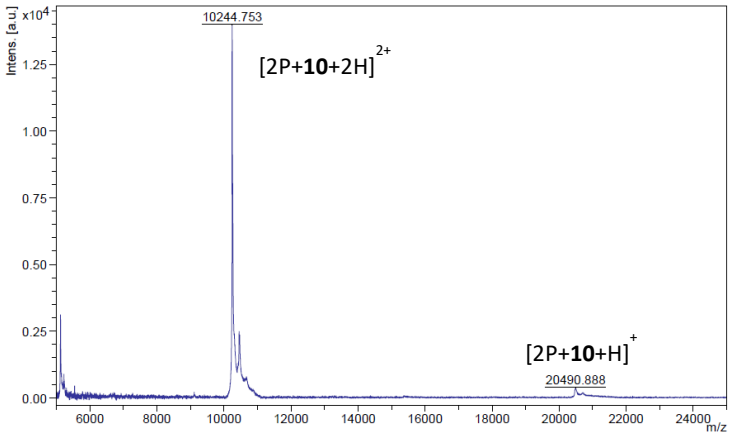


Figure 2. MALDI-ToF mass spectrum of the ionic complex formed between $(Arg\text{-}Gly)_{15}$ and dendrimer **10**. "P" means the $(Arg\text{-}Gly)_{15}$ peptide.

Langerin interaction studies by Surface Plasmon Resonance (SPR)

After completing the synthesis and characterization of second-generation CS-E dendrimers, we studied their interaction with Langerin by SPR experiments. Langerin is a transmembrane C-type lectin that is functionally active in a trimeric form. It can interact with GAGs, such as heparin and CS-E,[3] through two different binding sites.[2, 27, 28] The first one is the typical Ca^{2+} dependent carbohydrate recognition domain (CRD) of C-type lectins and is involved in the interaction with GAG oligosaccharides of small size. The second one is a Ca^{2+} independent site, located at the

positively charged groove formed between two adjacent CRD domains in the trimer. This Ca^{2+} independent site is particularly relevant when considering long GAG sequences. Therefore, we performed our SPR assays under two conditions, in the presence or absence of Ca^{2+}, in order to examine the effect of this cation on the interaction.

We calculated the relative binding affinity values for the different glycodendrimers by carrying out competition experiments.[2] For comparison purposes, we also included in this study monovalent CS-E disaccharide **11** and first-generation dendrimers **12**-**14** with valence 3, 4 and 6, respectively (Figure 3).[13] To perform these competition experiments, biotinylated 6 kDa heparin was first immobilized on a precoated Streptactin sensor chip, following a previously reported procedure.[2] Langerin was preincubated with a series of increasing concentrations of glycodendrimers and the resulting mixtures were then injected onto the heparin functionalized chip. Thus, we could estimate the ability of our multivalent systems to compete with immobilized heparin for Langerin binding. The analysis of the obtained sensorgrams (see Supporting Information) afforded relative binding affinities, expressed as IC_{50} values, for each tested CS-E dendrimer (Table 2). We have performed these studies in the presence of Ca^{2+} as well as in absence of this cation (sequestering this atom by adding EDTA). The results indicated that our synthetic multivalent systems were able to interact with Langerin, in the presence or absence of Ca^{2+}, in the micromolar range. As mentioned before, DLS measurements showed that second-generation glycodendrimers did not aggregate at 150 μM, a concentration above the obtained IC_{50}'s, both in the presence and the absence of Ca^{2+}. Therefore, we could discard the formation of aggregates of derivatives **6**, **8** and **10** under the conditions used for the SPR binding assays. In addition, monovalent disaccharide **11** showed significantly higher IC_{50} values confirming that the multivalent presentation of this epitope on dendritic cores strongly enhanced

its recognition by the lectin. On the other hand, the IC_{50} values for all the compounds in the presence of Ca^{2+} were higher than those obtained in the absence of this cation (with EDTA). This fact suggested that CS-E dendrimers compete more efficiently with immobilized heparin for the Ca^{2+} independent binding site of Langerin. Interestingly, in the presence of Ca^{2+}, the binding affinities of CS-E glycodendrimers generally increased with valence, showing modest relative inhibitory potency (RIP) values. Thus, second-generation glycodendrimers **8** and **10** gave the lowest IC_{50} values. In contrast, we did not observe a clear trend between IC_{50}'s and valence in the absence of calcium, indicating that not only the valence but also the nature of the dendritic core influence on the Ca^{2+} independent interaction of these systems with Langerin. In the presence of EDTA, second-generation dendrimers **6** and **8** afforded the strongest binding affinities (IC_{50} = 8.7 and 9.2 μM, respectively). The high RIP value obtained for nonavalent dendrimer **6** (and also for trivalent compound **12**) suggested that the presence of the central aromatic ring in the dendritic core may favor the molecular recognition of this derivative by Langerin.

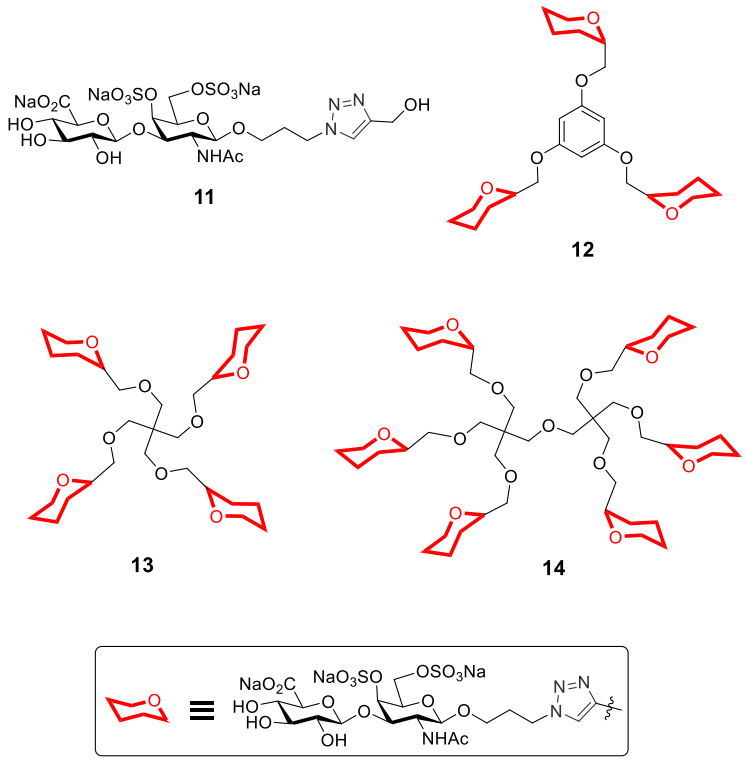
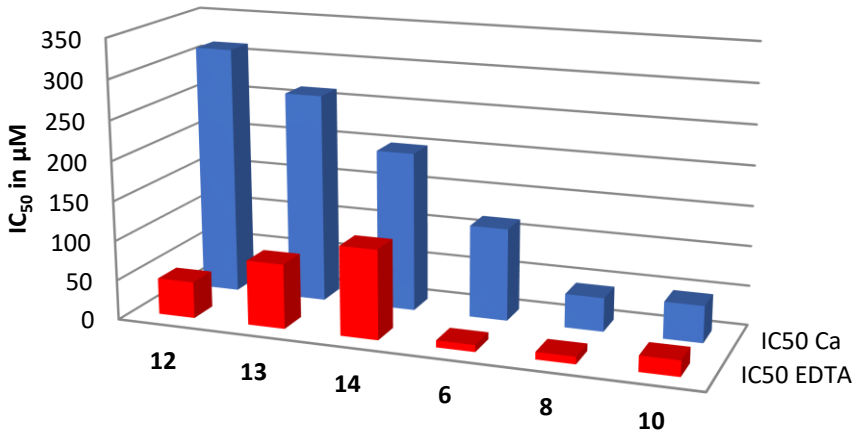


Figure 3. Structures of monovalent CS-E disaccharide **11** and first-generation glycodendrimers **12**-**14**.



Compounds	IC_{50} in Ca^{2+} buffer, µM	IC_{50} in EDTA buffer, µM	RIP in Ca^{2+} buffer	RIP in EDTA buffer
11	2470	1397	1	1
12	313.9	45.8	2.6	10.2
13	263.7	81.3	2.3	4.3
14	200.4	112.2	2.1	2.1
6	116.4	8.7	2.4	17.8
8	42.4	9.2	4.9	12.6
10	46.2	19.8	3.0	3.9

Table 2. IC_{50} values obtained from SPR inhibition experiments of Langerin ECD/heparin interaction by the various compounds. Langerin concentration was fixed at 20 µM concentration and mixed with increasing compound concentrations with 2-fold serial dilution factor. Relative inhibitory potency (RIP) values were calculated as $(IC_{50})_{\text{monovalent } \mathbf{11}}/(IC_{50} \times \text{valence})$.

Conclusions

In this work, we have shown a straightforward synthesis of second generation dendrimers bearing up to 18 copies of a CS-E disaccharide. These compounds were characterized by NMR and MALDI-Tof MS. In the case of the MS analysis, the formation of complexes with positive charged proteins or peptides was required to analyze these dendrimers facilitating that these complexes can fly under the MS conditions. This characterization demonstrated the homogeneity of the samples and their complete monodispersity. The capacity of these glycodendrimers to interact with a relevant human lectin, Langerin, involved in the mechanism of the organism defense against viral infections, was evaluated. By SPR competition studies, we have demonstrated that the higher multivalent dendrimers were capable to interact efficiently with Langerin and that they mainly do so through the Ca^{2+} independent binding site, efficiently competing with the natural polysaccharide heparin. Overall, our results highlight the utility of our second-generation CS-E dendrimers, bearing well-defined sulfation patterns, as excellent tools to mimic the biological interactions of the natural polysaccharide.

ASSOCIATED CONTENT

Supporting Information.

The following file is available free of charge.

MALDI-ToF mass spectrum of FGF-2, NMR spectra of new compounds, DLS measurements, SPR sensorgrams and inhibition curves (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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