PAMAM-based mannose-glycodendrimers as multi-electron redox probes for improving lectin sensing

*Manuel C. Martos-Maldonado, Juan M. Casas-Solvas, Indalecio Quesada-Soriano, Luis García-Fuentes, and Antonio Vargas-Berenguel**

Department of Chemistry and Physics, University of Almería, Carretera de Sacramento s/n, 04120 Almería, Spain.

ABSTRACT: An easy-to-prepare series of electroactive PAMAM-based dendrimers of generations G0 to G2 having mannopyranosylferrocenyl moieties in the periphery to detect carbohydrate-protein interactions is reported. The synthesis involved the functionalization of the PAMAM surface with azidomethylferrocenyl groups and subsequent coupling of mannoside units by Cu(I)-catalyzed Huisgen reaction. The binding affinity of the series of electroactive glycodendrimers was studied by isothermal titration calorimetry (ITC) and differential pulse voltammetry (DPV). Upon complexation of the glycodendrimers conjugates with prototypical concanavalin A (Con A), voltammograms showed a decrease of the peak current. Such dendrimers showed a notable improvement of redox sensing abilities towards Con A when compared with mono- and divalent analogues, based on both the glycoside multivalent and ferrocene dendritic effects.

INTRODUCTION: The binding interactions between carbohydrates and cell-surface proteins govern important biological events such as viral and bacterial infections, cell-cell adhesion, inflammatory and immune response, fertilization, and cancer metastasis.[1,2] The understanding of such events requires the development of synthetic tools which can be used to inhibit, modulate, detect and probe carbohydrate-proteins interactions.[3] The most common analytical techniques used for studying the interactions between carbohydrates and proteins such as lectins include affinity assays, fluorescence spectroscopy, calorimetry, surface plasmon resonance and quartz crystal microbalance. For some of techniques the use of fluorophore- or biotin-labelled proteins or carbohydrates is required, so that the recognition event induces a measurable signal.[4-10] The development of electrochemical methods for detection of lectin-glycans is receiving increase attention.[10] There are advantages attributed to electrochemical biosensors such as their low cost and high sensitivity, the fact that they are more susceptible to miniaturization and they are well suited for operating in turbid media.[11,12] A particular feature of the carbohydrate-protein binding interactions is a low affinity as compared, for example, with protein-protein binding interactions.[13-15] To overcome such weak affinity, cell-surface carbohydrates appear arranged as multivalent clusters and thus allow for multivalent interactions to take place and achieve high affinity due to the glycoside multivalent effect.[16,17] Different synthetic strategies such as glycoclusters, glycopolymers, glycodendrimers and glyconanoparticles have been followed to prepare multivalent glycoconjugates with enhanced affinity to lectins.[5,18-24] Thus, multivalent carbohydrate structures labelled with an optical or electrochemical moiety can be used for probing carbohydrate-lectin recognition.[8-10,25,26] In a different context, dendrimers with multiple copies of redox units on their surfaces can function as redox sensors, molecular batteries and catalysts, among other applications.[27-29] In particular, the electrochemical behaviour of ferrocenyl

dendrimers is characterized by a single voltammetric wave due to the "near" equivalence of the redox potential of each ferrocene moieties. In addition, multiple ferrocene moieties provide an amplified electrochemical signal due to its dependence on the number of electrons exchanged.[30] We have shown that mannose-ferrocene conjugates are electrochemical probes for the detection of lectin Concanavalin A (Con A).[25] In a further step, we envisaged that a way to improve the sensing abilities of such conjugates could take advantage of both the glycoside multivalent effect[13-17] and ferrocene dendritic effect.[31-34] Thus, we designed redox glycodendrimers consisting of multiple units of ferrocene-mannose conjugates as electrochemical sensors with improved sensing abilities. There are very few examples of electroactive glycodendrimers that can be used to sense lectins. However, in such reported cases multivalency is referred only to carbohydrates since the described dendrimers do not contain multiple units of electroactive moieties.[26] Herein, we report a convenient synthesis of electroactive PAMAM-based glycodendrimers from generation G0 to G2 functionalized in the periphery with ferrocene-mannose units and their abilities to function as electrochemical sensors towards Con A as a model lectin.

EXPERIMENTAL SECTION:

General Methods: TLC was performed on Merck Silica Gel 60 F254 aluminium sheets and developed by UV light and ethanolic sulfuric acid (5 % v/v). Flash column chromatography was performed on Merck Silica Gel (230-400 mesh, ASTM). Melting points were measured on a Büchi B-450 melting point apparatus and are uncorrected. Optical rotations were recorded on a Jasco P-1030 polarimeter at room temperature. $[\alpha]_D$ values are given in 10^{-1} deg· cm^2· g^{-1}. IR spectra were recorded on a Mattson Genesis II FTIR. ^1H, ^{13}C and 2D NMR spectra (gCOSY and gHMQC) were recorded on a Bruker Avance DPX300 spectrometer equipped with a QNP

$^{1}H/^{13}C/^{19}F/^{31}P$ probe. Standard Bruker software was used for acquisition and processing routines. Chemical shifts are given in ppm and referenced to internal TMS (δ_H and δ_C 0.00). *J* values are given in Hz. ESI-TOF mass spectra were recorded on a VG Analytical Quattro spectrometer and Waters Xevo Qtof spectrometer. HRFAB mass spectra were recorded on a Micromass Autospec spectrometer using glycerol as matrix. MALDI-TOF mass spectra were recorded on a Perspective Biosystems Voyager DR-RP spectrometer using 2,5-dihydroxybenzoic acid as matrix. Concanavalin A lectin (type VI, lyophilised powder) and other reagents were purchased from Aldrich and used as received. 1-(Azidomethyl)-1'-(methoxycarbonyl)ferrocene (**1**),[35] propargyl α-D-mannopyranoside (**9**), [4-(α-D-mannopyranosyloxymethyl)-1H-1,2,3-triazol-1-yl]methyl ferrocene (**13**) and 1,1'-bis{[4-(α-D-mannopyranosyloxymethyl)-1H-1,2,3-triazol-1-yl]methyl}ferrocene (**14**) were prepared as reported.[36] Solvents were dried according to literature procedures.[37] The concentration of the lectin solutions was determined by spectrophotometry ($A^{1\%}_{280\ nm}$=13.7 for the tetrameric form). All solutions for calorimetric and voltammetric studies were made in distilled and deionized water (MilliQ, 18.2 MΩ cm).

Voltammetric measurements: Electrochemical measurements were carried out in sonicated, nitrogen-purged H_2O (MilliQ 18.2 MΩ cm) solutions with a μAutolab type III potentiostat connected to a Intel Pentium Dual CPU 2.4 GHz personal computer running Eco Chimie B. V. GPES 4.9.5 software. The electrodes were carefully cleaned before each experiment. The platinum disk working electrode (Ø 3 mm, effective area 0.075 ± 0.002 cm^2) was immersed in a 50% v/v H_2SO_4 solution for 5 min. The glassy carbon disk counter electrode (Ø 2 mm, effective area 0.038 ± 0.006 cm^2) was immersed in a 0.1 M HNO_3 solution for 5 min and polished with a basic Al_2O_3–water slurry. Both electrodes were then sonicated in a 1:1:1 H_2O–MeOH–CH_3CN mixture for 5 min prior to use. The effective area of the electrodes was determined as previously

reported.[36,38] A Ag/AgCl (3 M KCl) electrode was used as a reference. Differential pulse voltammetry (DPV) experiments were carried out in 10 mM TRIS buffer (pH 7.2) with 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$ and 200 mM NaCl for **10**, **11**, **13** and **14**, and with 20 mM NaCl for **12**. Solutions of each conjugate (50 μM for **10**, **13**, **14**, 25 μM for **11** and 12.5 μM for **12**) and increasing amounts of Concanavalin A varying from 0 to 50 μM were prepared in this buffer and incubated for 1 h at room temperature. Before each experiment, nitrogen was bubbled for 3 min. A DPV experiment was then measured between 0 V and + 0.7 V for **13** and **14**, 0 V and + 0.9 V for **10**-**12**, with a scan rate of 5 mV s^{-1}, a step potential of 20 mV, a modulation amplitude of 50 mV, a modulation time of 0.05 s and an interval time of 2 s. The voltammetric data were fit to an equal and independent sites binding model algorithm implemented by the authors[39] by using 'Scientist' software (Micromath Scientific Software, St. Louis, USA) to determine the binding constant *K*.

Isothermal titration calorimetry measurements: Calorimetric experiments were conducted using either a MCS or an ultrasensitivity VP-ITC (Microcal Inc., Northampton, MA). The sample preparation and ITC experiments were carried out as previously described elsewhere.[40] Titrations were routinely performed in 10 mM TRIS buffer (pH 7.2) with 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$ and 20 mM NaCl. Blank titrations of ligand into buffer were also performed to correct the heat generated by dilution and mixing. An equal and independent sites model (non-cooperative model) has been used to fit the experimental data. The experimental data were fitted using 'Scientist' software (Micromath Scientific Software, St. Louis, USA) to the model algorithm implemented by us. The equations used in these models have been widely described in literature.[41] The changes in the standard free energy ΔG^0 and entropy ΔS^0 were determined as $\Delta G^0 = -RT\ln K$ and $T\Delta S^0 = \Delta H - \Delta G^0$ (assuming that $\Delta H = \Delta H^0$).

1'-(Azidomethyl)ferrocenoic acid (2). Water (40 μL, 2.222 mmol) and sodium *tert*-butoxide (300 mg, 2.673 mmol) were added in this order to a solution of 1-(azidomethyl)-1'-(methoxycarbonyl)ferrocene (**1**)[35] (672 mg, 2.247 mmol) in THF (12 mL) under N_2 atmosphere. The mixture was stirred at room temperature for 2 h in the darkness. After that, the reaction was cooled to 0 ºC and acidified until pH 2-3 with diluted $HCl_{(aq.)}$. The aqueous solution was extracted with ethyl acetate (3 × 50 mL). The organic layer was dried with $MgSO_4$ and filtered. The solvent was removed by evaporation under vacuum and the crude was purified by column chromatography (hexane-Et_2O 1:2) to yield **2** (525 mg, 82 %) as an orange solid. M. p. 99 ºC; IR (KBr, cm^{-1}): 2947, 2864, 2637, 2557, 2094, 1663, 1482, 1375, 1332, 1302, 1260, 1167, 1026, 934, 912, 834, 815, 743, 561, 492, 417; δ_H (300 MHz, $CDCl_3$): 10.2 (bs, 1H, CO_2H), 4.87 (t, 2H, ^3J = 2.0 Hz, H_{Cp}), 4.50 (t, 2H, ^3J = 2.0 Hz, H_{Cp}), 4.31 (m, 4H, H_{Cp}), 4.13 (s, 2H, CH_2N_3); δ_C (75 MHz, $CDCl_3$): 178.1 (CO), 83.6 (C_{ipso}), 72.8 (C_{Cp}), 71.5 (C_{Cp}), 70.8 (C_{Cp}), 70.6 (C_{ipso}), 70.6 (C_{Cp}), 49.8 (CH_2N_3); *m*/*z* (HRFAB): Calc. for $C_{12}H_{11}FeN_3O_2$ 285.0201. Found: 285.0198 $[M]^+$.

General procedure for the synthesis of azidomethyl-ferrocenylated PAMAM dendrimers 6-8. The amine-terminated PAMAM dendrimers **3-5** were received as solutions in methanol, and prior to use the solvent was removed in vacuum. After that, the resulting material and 1'-(azidomethyl)ferrocenoic acid (**2**, 1.5 eq. per amino group) were dissolved in dry DMF (1 mL per 200 mg of **2**) under N_2 atmosphere. Then, 1-hydroxybenzotriazole (HOBt, 1.6 eq. per amino group), *N*,*N*'-dicyclohexylcarbodiimide (DCC, 1.6 eq. per amino group) and Et_3N (2 eq. per amino group) were added to the solution in this order. The resulting mixture was stirred at room temperature in the darkness for 3 days. The crude was diluted with $CHCl_3$ (10 mL per 1 mL of DMF) and filtered. Hexane was added to the filtrate until an orange precipitate appeared, which was filtered off and purified by column chromatography.

G0-PAMAM-($FcCH_2N_3$)$_4$ (6). Starting from **3** (81.2 mg, 0.157 mmol) the column chromatography (CH_2Cl_2-MeOH 10:2) yielded **6** (161 mg, 65 %) as an orange viscous oil. IR (KBr, cm^{-1}): 3300, 3084, 2986, 2941, 2098, 1643, 1545, 1450, 1398, 1385, 1302, 1266, 1192, 1099, 1030, 837, 746, 513; δ_H (300 MHz, $CDCl_3$): 7.85 (bs, 4H, NH), 7.46 (bs, 4H, NH), 4.79 (bs, 8H, H_{Cp}), 4.36 (bs, 8H, H_{Cp}), 4.24 (m, 16H, H_{Cp}, CH_2N_3), 4.09 (bs, 8H, H_{Cp}), 3.52-3.37 (m, 16H, C*H*$_2$NHCO), 2.63 (bs, 8H, CH_2N), 2.39 (bs, 4H, CH_2N), 2.31 (bs, 8H, CH_2CO); δ_C (75 MHz, $CDCl_3$): 173.8 (CO), 171.1 (CO), 83.4 (C_{ipso}), 77.4 (C_{ipso}), 71.5 (C_{Cp}), 70.6 (C_{Cp}), 70.3 (C_{Cp}), 69.2 (C_{Cp}), 50.9 (CH_2N), 50.1 (CH_2N), 50.1 (CH_2N_3), 40.5 (*C*H$_2$NHCO), 39.6 (*C*H$_2$NHCO), 33.8 (*C*H$_2$CO); *m*/*z* (MALDI-TOF): Calc. for $C_{70}H_{84}Fe_4N_{22}O_8$ 1584.4. Found: 1585.3 $[M + H]^+$, 1607.3 $[M + Na]^+$.

G1-PAMAM-($FcCH_2N_3$)$_8$ (7). Starting from **4** (124 mg, 0.087 mmol) the column chromatography (CH_2Cl_2-MeOH 10:2 →CH_2Cl_2-MeOH-Et_3N 80:15:10) yielded **7** (186 mg, 60 %) as an orange viscous oil. IR (KBr, cm^{-1}): 3298, 3084, 2981, 2941, 2098, 1651, 1546, 1450, 1398, 1382, 1302, 1263, 1192, 1099, 1040, 837, 746, 595, 513; δ_H (300 MHz, $CDCl_3$): 8.03 (bs, 8H, NH), 7.89 (bs, 4H, NH), 7.80 (bs, 8H, NH), 4.83 (bs, 16H, H_{Cp}), 4.33 (bs, 16H, H_{Cp}), 4.21 (m, 32H, H_{Cp}, CH_2N_3), 4.07 (bs, 16H, H_{Cp}), 3.53-3.30 (m, 32H, C*H*$_2$NHCO), 3.17 (bs, 8H, C*H*$_2$NHCO), 3.10-2.60 (m, 28H, CH_2N), 2.48 (bs, 4H, CH_2N), 2.42-2.30 (bs, 24H, CH_2CO); δ_C (75 MHz, $CDCl_3$): 173.8 (CO), 171.2 (CO), 171.1 (CO), 83.3 (C_{ipso}), 77.4 (C_{ipso}), 71.5 (C_{Cp}), 70.6 (C_{Cp}), 70.4 (C_{Cp}), 69.3 (C_{Cp}), 52.5 (CH_2N), 50.6 (CH_2N), 50.1 (CH_2N_3), 48.7 (CH_2N), 40.1 (*C*H$_2$NHCO), 39.8 (*C*H$_2$NHCO), 37.8 (*C*H$_2$NHCO), 33.8 (*C*H$_2$CO), 31.6 (*C*H$_2$CO); *m*/*z* (ESI-TOF): Calc. for $C_{158}H_{200}Fe_8N_{50}O_{20}$ 3566.10. Found: 3567.02 $[M + H]^+$, 1795.03 $[M + H + Na]^{2+}$, 1784.04 $[M + 2H]^{2+}$.

G2-PAMAM-(FcCH$_2$N$_3$)$_{16}$ (8). Starting from **5** (97 mg, 0.030 mmol) the column chromatography (CH_2Cl_2-MeOH 10:2 →CH_2Cl_2-MeOH-Et_3N 80:20:10) yielded **8** (147 mg, 65 %) as an orange viscous oil. IR (KBr, cm^{-1}): 3298, 3084, 2981, 2941, 2098, 1651, 1547, 1450, 1398, 1383, 1302, 1263, 1192, 1099, 1041, 837, 746, 596, 513; δ_H (300 MHz, $CDCl_3$): 8.03 (bs, 20H, NH), 7.79 (bs, 24H, NH), 4.81 (s, 32H, H_{Cp}), 4.33 (s, 32H, H_{Cp}), 4.22 (m, 64H, H_{Cp}, CH_2N_3), 4.08 (s, 32H, H_{Cp}), 3.55-3.34 (m, 64H, C*H*$_2$NHCO), 3.19 (bs, 24H, C*H*$_2$NHCO), 2.81-2.60 (m, 60H, CH_2N), 2.48 (bs, 24H, CH_2N), 2.42-2.20 (m, 56H, CH_2CO); δ_C (75 MHz, $CDCl_3$): 173.8 (CO), 172.8 (CO), 171.6 (CO), 170.9 (CO), 83.1 (C_{ipso}), 77.4 (C_{ipso}), 71.4 (C_{Cp}), 70.6 (C_{Cp}), 70.3 (C_{Cp}), 69.2 (C_{Cp}), 52.4 (CH_2N), 50.5 (CH_2N), 50.0 (CH_2N_3), 48.9 (CH_2N), 40.2 (*C*H$_2$NHCO), 39.6 (*C*H$_2$NHCO), 37.6 (*C*H$_2$NHCO), 33.8 (*C*H$_2$CO), 31.6 (*C*H$_2$CO); *m*/*z* (ESI-TOF): Calc. for $C_{334}H_{432}Fe_{16}N_{106}O_{44}$ 7526.44. Found: 2532.45 $[M + 3Na]^{3+}$, 2525.80 $[M + H + 2Na]^{3+}$, 2518.14 $[M + 2H + Na]^{3+}$, 2510.48 $[M + 3H]^{3+}$.

General procedure for the synthesis of electroactive mannosylated PAMAM dendrimers 10-12. The azide-functionalized PAMAM dendrimers (**6**-**8**) and propargyl α-D-mannopyranoside (**9**, 1.2 eq. per azide group) were dissolved in dry DMF (1 mL per 50 mg of **9**) under N_2 atmosphere. Then, CuI· EtO_3P (0.2 eq. per azido group) and DIPEA (2 eq. per azido group) were added to the solution in this order. The resulting mixture was stirred at room temperature in the darkness for 24 hours. After that the crude was poured into acetone (50 mL) and the resulting precipitate was filtered. Then, the solid was dissolved in water and the solvent was removed by lyophilization. The resulting yellow solid was purified by repetitive precipitation in methanol.

G0-PAMAM-(FcCH$_2$TACH$_2$Man)$_4$ (10). Starting from **6** (100 mg, 0.063 mmol) the general procedure yielded **10** (90 mg, 58 %) as a yellow solid. M. p. 155 ºC (dec.); $[\alpha]_D$ +48º (*c* 0.2, DMSO); IR (KBr, cm^{-1}): 3362, 2934, 1634, 1545, 1450, 1400, 1383, 1306, 1236, 1128, 1101,

1056, 818, 748, 685, 596, 513, 492 ; δ_H (300 MHz, DMSO-*d*$_6$): 8.09 (bs, 4H, NH), 8.06 (s, 4H, H-5-C_2HN_3), 8.01 (bs, 4H, NH), 5.23 (s, 8H, CH_2N_3), 4.82 (s, 8H, H_{Cp}), 4.74 (bs, 8H, OH), 4.69 (s, 4H, H-1), 4.62 (d, 4H, ^2J = 12.0 Hz, CHO), 4.58-4.50 (m, 8H, OH), 4.46 (d, 4H, ^2J = 12.0 Hz, CHO), 4.36 (s, 8H, H_{Cp}), 4.32 (m, 8H, H_{Cp}), 4.16 (s, 8H, H_{Cp}), 3.67 (d, 4H, $^3J_{6,6'}$ = 9.0 Hz, H-6), 3.54 (s, 4H, H-2), 3.51-3.30 (m, H-3,4,5,6', HDO), 3.29-3.10 (m, 16H, C*H*$_2$NHCO), 2.64 (bs, 8H, CH_2N), 2.42 (bs, 4H, CH_2N), 2.21 (bs, 8H, CH_2CO); δ_C (75 MHz, DMSO-*d*$_6$): 171.8 (CO), 168.8 (CO), 143.6 (C-4-C_2HN_3), 123.5 (C-5- C_2HN_3), 99.0 (C-1), 82.7 (C_{ipso}), 77.3 (C_{ipso}), 74.1 (C-5), 70.9 (C_{Cp}), 70.2 (C_{Cp}, C-3), 70.1 (C_{Cp}, C_{Cp}, C-2), 68.9 (C_{Cp}), 67.0 (C-4), 61.3 (C-6), 59.1 (CH_2O), 50.9 (CH_2N), 49.7 (CH_2N), 48.2 (CH_2N_3), 39.2 (*C*H_2NHCO), 38.2 (*C*H_2NHCO), 33.2 (*C*H_2CO); *m*/*z* (MALDI-TOF): Calc. for $C_{106}H_{140}Fe_4N_{22}O_{32}$ 2457.74. Found: 2481.74 $[M + Na + H]^+$, 2459.64 $[M + 2H]^+$. *m*/*z* (HRESI-TOF): Calc. for $C_{106}H_{140}Fe_4N_{22}O_{32}$ 2457.7435. Found: 2458.8955 $[M + H]^+$.

G1-PAMAM-(FcCH$_2$TACH$_2$Man)$_8$ (11). Starting from **7** (95 mg, 0.026 mmol) the general procedure yielded **11** (71 mg, 50 %) as a yellow solid. M. p. 171 ºC (dec.); $[\alpha]_D$ +51º (*c* 0.2, DMSO); IR (KBr, cm^{-1}): 3317, 2928, 1636, 1543, 1452, 1435, 1379, 1304, 1236, 1130, 1055, 816, 682, 588, 513, 490 ; δ_H (300 MHz, DMSO-*d*$_6$): 8.15-7.94 (m, 28H, NH, H-5-C_2HN_3), 5.22 (s, 16H, CH_2N_3), 4.81 (s, 16H, H_{Cp}), 4.80-4.71 (m, 16H, OH), 4.69 (s, 8H, H-1), 4.62 (d, 8H, ^2J = 12.0 Hz, CHO), 4.58-4.50 (m, 16H, OH), 4.46 (d, 8H, ^2J = 12.0 Hz, CHO), 4.35 (s, 16H, H_{Cp}), 4.31 (m, 16H, H_{Cp}), 4.16 (s, 16H, H_{Cp}), 3.67 (d, 8H, $^3J_{6,6'}$ = 9.0 Hz, H-6), 3.55 (s, 8H, H-2), 3.50-3.29 (m, H-3,4,5,6', HDO, 3.29-3.11 (m, 32H, C*H*$_2$NHCO), 3.10 (bs, 8H, C*H*$_2$NHCO), 2.69-2.60 (m, 28H, CH_2N), 2.44 (bs, 8H, CH_2N), 2.31-2.12 (bs, 24H, CH_2CO); δ_C (75 MHz, DMSO-*d*$_6$): 171.8 (CO), 171.3 (CO), 168.8 (CO), 143.6 (C-4- C_2HN_3), 123.5 (C-5-C_2HN_3), 99.0 (C-1), 82.7 (C_{ipso}), 77.3 (C_{ipso}), 74.1 (C-5), 70.9 (C_{Cp}), 70.2 (C_{Cp}, C-3), 70.1 (C_{Cp}, C_{Cp}, C-2), 69.0 (C_{Cp}), 67.0

(C-4), 61.3 (C-6), 59.0 (CH_2O), 52.0 (CH_2N), 49.6 (CH_2N), 48.2 (CH_2N_3), 39.2 (*C*H_2NHCO), 38.9 (*C*H_2NHCO), 37.3 (*C*H_2NHCO), 33.3 (*C*H_2CO). *m*/*z* (HRESI-TOF): Calc. for $C_{230}H_{312}Fe_8N_{50}O_{68}$ 5311.7356. Found: 1328.9999 $[M + 4H]^{4+}$. Number of expected mannose units per dendrimer: 8. Found by sulfuric acid-phenol assays: 7.90 ± 0.17 (see Supporting Information).

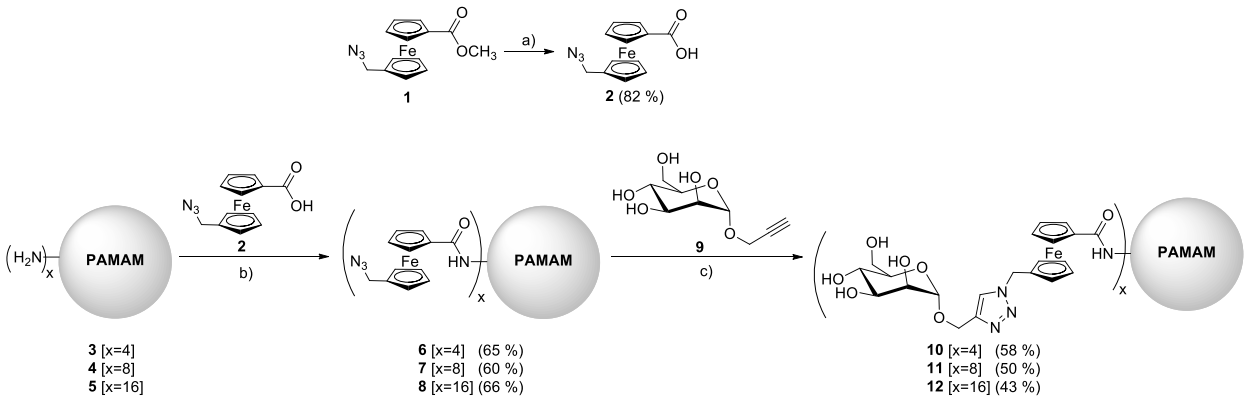
G2-PAMAM-(FcCH$_2$TACH$_2$Man)$_{16}$ (12). Starting from **8** (115 mg, 0.015 mmol) the general procedure yielded **12** (72 mg, 43 %) as a yellow solid. M. p. 163 ºC (dec.); $[\alpha]_D$ +56º (*c* 0.2, DMSO); IR (KBr, cm^{-1}): 3339, 2928, 1638, 1547, 1452, 1435, 1381, 1306, 1236, 1130, 1055, 814, 682, 588, 490 ; δ_H (300 MHz, DMSO-*d*$_6$): 8.11-7.90 (m, 60H, NH, H-5-C_2HN_3), 5.24 (s, 32H, CH_2N_3), 4.82 (s, 32H, H_{Cp}), 4.80-4.70 (m, 32H, OH), 4.71 (s, 16H, H-1), 4.59 (d, 16H, ^2J = 12.0 Hz, CHO), 4.58-4.50 (m, 32H, OH), 4.47 (d, 16H, ^2J = 12.0 Hz, CHO), 4.36 (s, 32H, H_{Cp}), 4.32 (m, 32H, H_{Cp}), 4.17 (s, 32H, H_{Cp}), 3.67 (d, 16H, $^3J_{6,6'}$ = 9.0 Hz, H-6), 3.55 (s, 16H, H-2), 3.50-3.30 (m, H-3,4,5,6', HDO), 3.29-3.14 (m, 64H, C*H*$_2$NHCO), 3.10 (bs, 24H, C*H*$_2$NHCO), 2.69-2.60 (m, 60H, CH_2N), 2.43 (bs, 24H, CH_2N), 2.31-2.12 (bs, 56H, CH_2CO); δ_C (75 MHz, DMSO-*d*$_6$): 171.8 (CO), 171.3 (CO), 168.8 (CO), 143.6 (C-4-C_2HN_3), 123.5 (C-5-C_2HN_3), 99.0 (C-1), 82.7 (C_{ipso}), 77.3 (C_{ipso}), 74.1 (C-5), 70.9 (C_{Cp}), 70.2 (C_{Cp}, C-3), 70.1 (C_{Cp}, C_{Cp}, C-2), 69.0 (C_{Cp}), 67.0 (C-4), 61.3 (C-6), 59.0 (CH_2O), 52.1 (CH_2N), 49.5 (CH_2N), 48.2 (CH_2N_3), 39.0 (*C*H_2NHCO), 38.6 (*C*H_2NHCO), 37.0 (*C*H_2NHCO), 33.3 (*C*H_2CO). Number of expected mannose units per dendrimer: 16. Found by sulfuric acid-phenol assays: 15.97 ± 0.24 (see Supporting Information).

RESULTS AND DISCUSSION:

Synthesis: For the construction of the electroactive glycodendrimers we first carried out the synthesis of a 1,1'-difunctional ferrocene building block having an azido and a carboxylic group (**2**, Scheme 1). The carboxylic group can be used for the coupling of **2** with the dendrimer through amide-bond formation. The azido group allows the coupling by Cu(I)-catalysed azide-alkyne cycloaddition of a carbohydrate of choice depending on the lectin receptor.[36,38] Building-block **2** was prepared from ferrocene derivative **1**[35] through the basic hydrolisys of the methyl ester. We used **2** to functionalize the PAMAM surface with azidomethylferrocenyl groups by amide coupling using DCC and HOBt yielding compounds **6-8** in 60 to 66% yields. The resulting azide-terminated PAMAM dendrimers were subsequently used in Cu(I)-catalyzed Huisgen reactions with propargyl mannopyranoside **9**[36] to obtain mannosylated electroactive dendrimers **10-12** in 43 to 58% yields. Compounds **10-12** were characterized by NMR spectroscopic techniques employing ^{1}H, ^{13}C and HMQC NMR experiments. The inspection of the ^{1}H-NMR spectra revealed the appearance of a signal at 8.15-7.90 ppm corresponding to the 1,2,3-triazole protons. HMQC spectra showed that the triazole methine carbon atoms resonate at 123.5 ppm. ^{13}C-NMR spectra also showed a signal at 143.6 ppm corresponding to the triazole C-4 atoms. Furthermore, ^{1}H-NMR spectra displayed a singlet at 5.22-5.23 ppm corresponding to the protons of the methylene that connect the triazol moieties with the ferrocene units. These observations confirmed the formation of the triazol rings. We also observed in the ^{13}C-NMR spectra a signal at 99.0 ppm corresponding to the anomeric carbon atoms of the sugar moieties. It is also worth of mention that the IR spectra of compounds **10-12** showed the absence of an intense band at 2098 cm^{-1} corresponding to the azide groups in compounds **6-8** (see Supporting Information). Finally, the molecular weight of compound **10** was verified by MALDI-TOF and

ESI-TOF mass spectrometry. Mass spectra of compound **11** and **12** were also recorded. However, MALDI-TOF spectra only showed broad peaks around the expected mass for both compounds. The ESI-TOF mass spectrum of compound **11** showed a peak that matched with $[M + 4H]^{4+}$. By contrast the ESI-TOF mass spectrum of compound **12** only showed peaks with a mass much more lower than the expected and did not match the multiply charge adducts, most likely due to multiple fragmentations of the molecule. A sulfuric acid-phenol assay was used to determine the number of mannose units per dendrimer in compounds **11** and **12** (see Supporting Information).

Scheme 1. Synthesis of the electroactive mannosylated-dendrimers **10**-**12**.[a] (see Chart 1 and Supporting Information for the complete detailed structure of dendrimers **6**-**8** and **10**-**12**)



[a] Reagents and conditions: (a) *t*-BuOK, H_2O, THF, r.t., 16 h. (b) DCC, HOBt, Et_3N, DMF, r.t., 72 h. (c) CuI· EtO_3P, DIPEA, DMF, r.t., 24 h.

Electrochemical characterization: The electrochemical properties of glycodendrimers **10**-**12** were studied by differential pulse voltammetry (DPV) in buffers containing Ca^{2+} and Mn^{2+} ions, which are required for the carbohydrate binding activity of the lectin.[2] In order to ensure a good conductivity in the solution and avoid electrical migration 200 mM NaCl was used as supporting

electrolyte for compounds **10** and **11**, but only 20 mM NaCl was used for **12** due to its lower solubility. Such concentration used for **12** still is 1.6×10^3 times higher than the concentration of the electroactive species. Glycodendrimers **10**-**12** showed only one oxidation peak at 0.560 V meaning that all ferrocene moieties are equivalent in the electrochemical time scale of the measurement. This is a typical electrochemical behavior observed for other ferrocenyl-dendrimers in organic solvents.[27-29, 31-34] DPV peak current (*I*) is directly proportional to the number of exchanged electrons, the electrode surface, the concentration of the electroactive species and the square of the diffusion coefficient of the electroactive species.[30] In our case, it is expected that an increase in the number of exchanged electrons would lead to an increase of the electrochemical signal. Such increase is partly compensated by the decrease of the peak current due to the slower diffusion of the species with increased molecular weight (Figure 1). For example, compound **12**, which has sixteen ferrocene residues, at 12.5 μM exhibits approximately 70% of the peak current measured for a four times more concentrated solution of tretraferrocene **10** (50 μM). Similarly, at the same concentration, **12** exhibits 84% of the peak current for a two times more concentrated solution of octaferrocene **11** (25 μM).

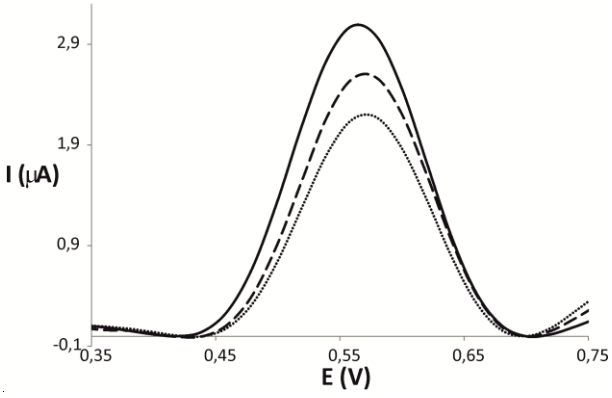
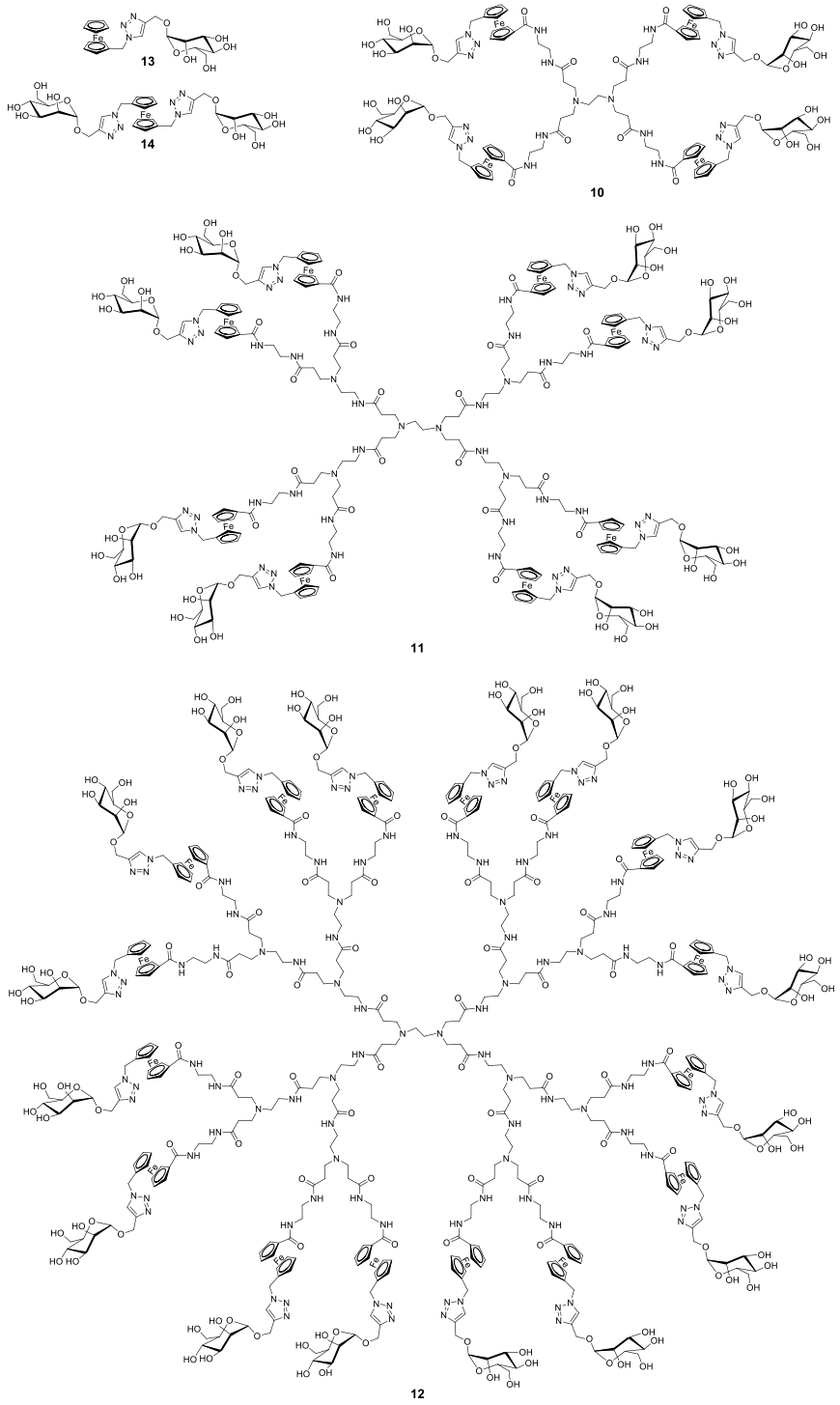


Figure 1. Differential pulse voltammograms for compound **10** (50 μM, solid line), **11** (25 μM, dashed line) and **12** (12.5 μM, dotted line) in 10 mM TRIS buffer (pH 7.2) with 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$ and 200 mM NaCl for **10** and **11**, and 20 mM NaCl for **12**.

Binding Studies: To study the binding abilities of **10**-**12** towards Con A, we carried out both isothermal titration calorimetry (ITC) and DPV measurements. The results were compared with the binding properties reported for mono-ferrocene mannose conjugates **13** and **14** (Chart 1) that contain 1 and 2 mannose units, respectively.[25] Both ITC and DPV experiments were developed at pH 7.2 where Con A exists as a tetramer that possesses four binding sites,[42] although the protein concentration was always expressed as monomer. In our case, the stepwise addition of aliquots of a solution of the glycoconjugates **10**-**12** into a solution containing Con A led to a decrease in the extent of released heat (Figure 2). The experimental data were fitted to an equal and independent sites model that allows the direct determination of the apparent binding constant between the dendrimers and the lectin (K), the binding enthalpy change (ΔH) and the [ligand]/[receptor] ratio in the saturation or stoichiometry (n).

Chart 1. Chemical structure of compounds **10-14**.



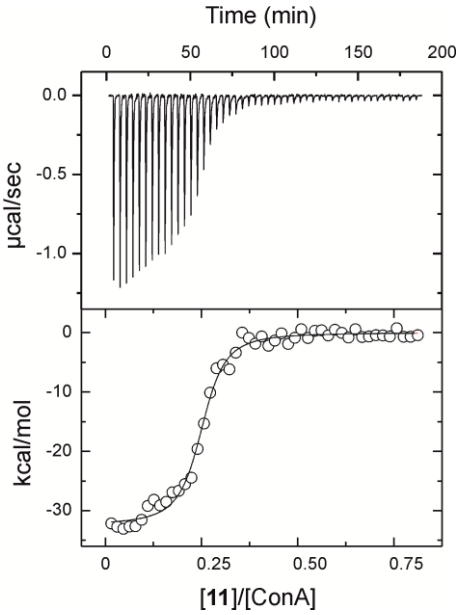


Figure 2. Titration of Con A with compound **11** in 10 mM TRIS buffer (pH 7.2) with 20 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ at 25 ºC. The top panel shows the raw calorimetric data denoting the amount of generated heat (negative exothermic peaks) following each injection of the solution of conjugate. The area under each peak represents the amount of heat released upon binding of the conjugate to the lectin. The smooth solid lines represent the best fit of the experimental data to an equal and independent sites model.

The analysis of the thermodynamic parameters showed that the complexations are enthalpy driven along with an unfavorable entropic contribution (Figure 3). An enthalpy-entropy compensation plot (Figure 4) showed a good linear correlation (r^2=1) with a similar slope (1.13) to those reported for the complexation of other mono- and multivalent mannosides with Con A.[43] In general, an increase in the magnitude of ΔH and $T\Delta S^0$ with the number of the mannoside residues of the glycoconjugates was observed (Figure 3). A deeper analysis revealed that ΔH is proportional to the number of mannoside units from monomer **13** to tetramer **10** (since ΔH values for dimer **14** and tetramer **10** are 2.4 and 4.4-fold higher than that for monomer **13**, respectively). However, this trend was not mantained for compounds **11** and **12** that present 8 and 16 mannose

residues but rather showed ΔH values 4.6 and 5.4-fold higher than that for monomer **13**, respectively. This observation suggests that not all mannoside units of **11** and **12** participate in the binding. This may be due to steric crowding, insufficient length of linkers and/or lack of spatial freedom of the carbohydrate components, resulting in insufficient participation in the binding.[44]

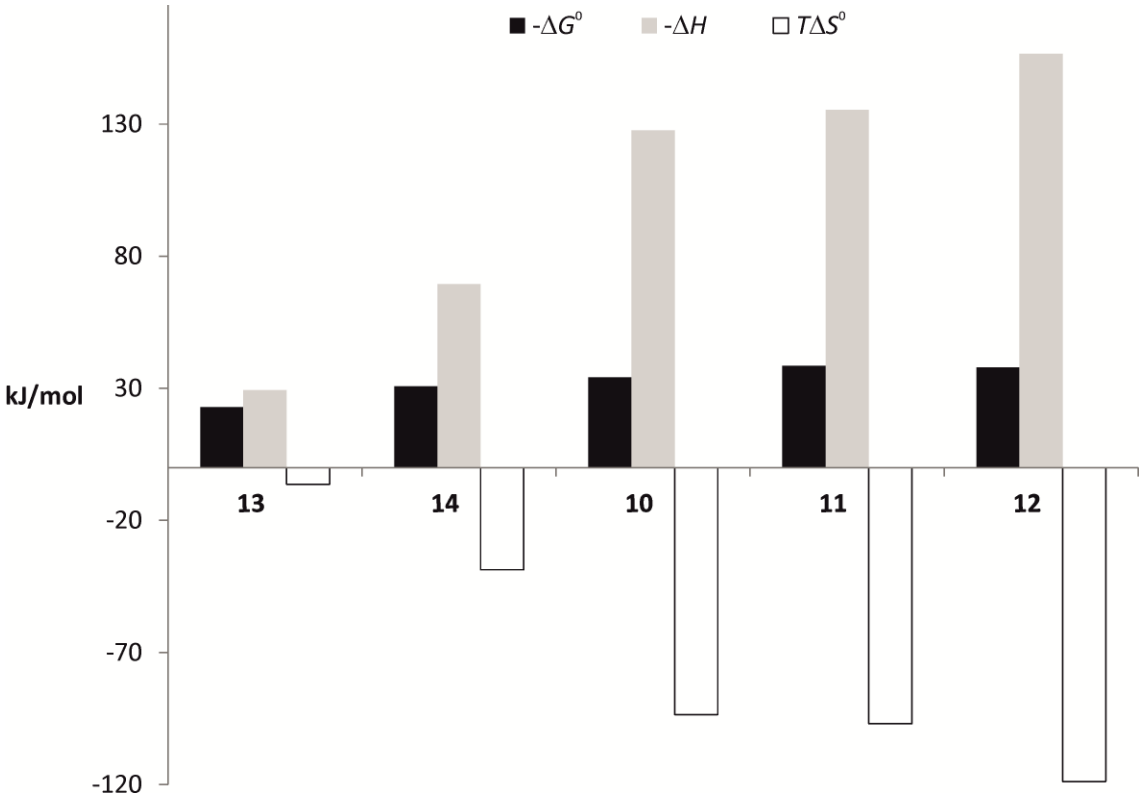


Figure 3. Free energy ($-\Delta G^0$, black), enthalpy ($-\Delta H$, grey), and entropy changes ($T\Delta S^0$, white) for the binding of compounds **10**–**14** to Con A obtained from ITC experiments at pH 7.2 and 25ºC.

In contrast, $T\Delta S^0$ does not increase proportionally with the number of mannoside residues in any case. For multivalent carbohydrate molecules where ΔH is proportional to the number of carbohydrate units but $T\Delta S^0$ is not, it has been proposed that the binding occurs with separated, unconnected receptor molecules instead of with a single multivalent receptor molecule.[42,43] This

suggests the formation of cross-linked complexes between the multivalent glycoconjugates and different protein units. The *n* values are <1 for all multivalent glycoconjugates (Table 1) as expected for the interactions of multivalent carbohydrates with a tetrameric lectin.[25,42] The *n* value became smaller as the valency of the glycoconjugate increased suggesting an increase of the cross-linking with the increase of the number of mannoside residues. While compounds **14** and **10** showed *n* values of 0.39 and 0.33, respectively, that are relatively close to the theoretically expected value for a dimer (1/2 = 0.5) and a tetramer (1/4 = 0.25), compounds **11** and **12** showed values of 0.25 and 0.23, respectively, farther away from the expected ones (1/8 = 0.125 for **11** and 1/16 = 0.063 for **12**) indicating that for these compounds not all of the mannoside residues are participating in the binding as pointed above.

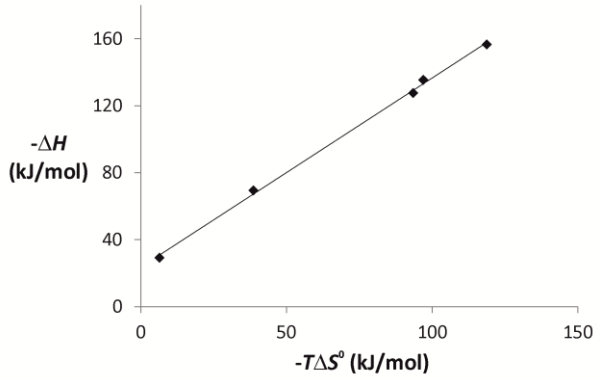


Figure 4. Plot of the $-\Delta H$ versus $-T\Delta S^0$ for the binding of compounds **10-14** to Con A.

Finally, as can be seen in Table 1, there is an increase of the *K* value with the number of mannose residues of the dendrimers from monomer **13** to octamer **11**, due to the multivalent effect. However, compound **12** that possesses sixteen mannose residues showed a *K* value slightly lower than that for octamer **11**. Taking into account that not all mannose moieties of these two conjugates are involved in the binding as referred above, this result suggests that the

ratio between the number of interacting sugars (which defines in great extent how negative the enthalpy term is) and the extension of the cross-linking (which mainly contributes to the negative entropy term) in the binding complex is more favorable in the latter Therefore, for the PAMAM scaffold the octameric glycodendrimer **11** possesses the optimum valency for high affinity binding to lectin.

Table 1. Thermodynamic parameters for compounds **10-14** obtained by isothermal titration calorimetry and voltammetry at pH 7.2 and 25ºC.

	Calorimetry					Voltammetry
Compound	$K\times10^{-5}$ (M^{-1})	$-\Delta G^0$ (kJ mol^{-1})	$-\Delta H$ (kJ mol^{-1})	$T\Delta S^0$ (kJ mol^{-1})	n	$K\times10^{-5}$ (M^{-1})
13	0.12 ± 0.01	7.14 ± 0.13	29.24± 1.37	-1.54 ± 0.12	1.00 (fix)	0.15 ± 0.01
14	2.47 ± 0.29	16.63 ± 1.32	69.46± 2.11	-9.32 ± 1.63	0.39 ± 0.07	0.25 ± 0.16
10	9.65 ± 1.11	30.51 ± 2.63	127.63± 2.34	-22.41± 2.63	0.33 ± 0.06	1.05 ± 0.41
11	57.21 ± 8.46	32.41 ± 2.52	135.42± 1.15	-23.24 ± 2.86	0.25 ± 0.08	11.52 ± 7.61
12	41.32 ± 5.65	37.56 ± 3.51	156.6 ± 3.12	-28.41 ± 2.73	0.23 ± 0.07	10.63 ± 5.21

DPV voltammograms displayed a progressive decrease of the peak current with the increase of lectin concentration (Figure 5). The glycoconjugate-lectin complexes are expected to diffuse much more slowly than the free uncomplexed glycoconjugate and since the peak current is proportional to the square root of the diffusion coefficient, these complexes have smaller peak current values than the latter. To obtain the binding constants, the experimental current data were fitted versus the concentration of Con A using an algorithm based on a model for equal and

independent binding sites.[39] Binding constants estimated by electrochemical methods showed the same trend as those obtained from ITC measurements (Table 1).

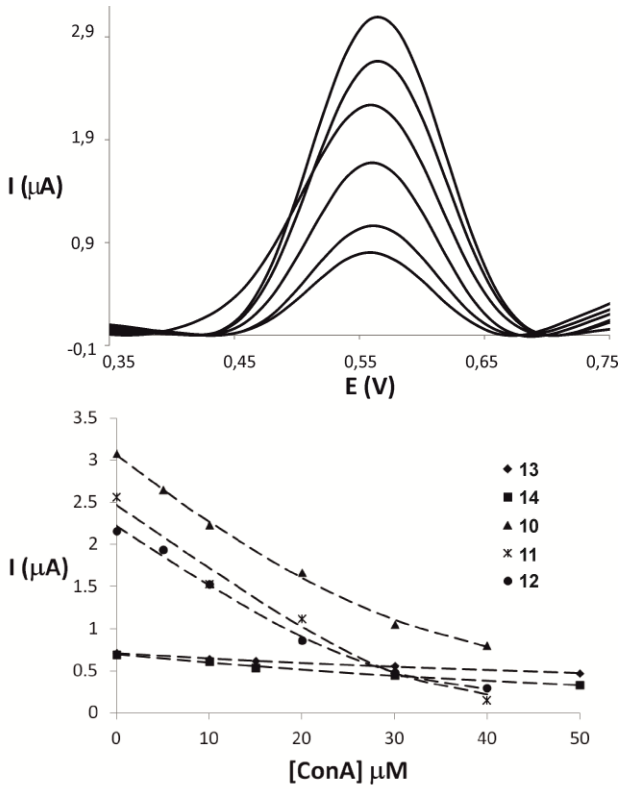


Figure 5. *Top*: Differential pulse voltammograms for compound **10** in the presence of increasing amounts of Con A (0-50 μM) in 10 mM TRIS buffer (pH 7.2) with 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$ and 200 mM NaCl. A decrease in the peak current was observed as Con A concentration increased. *Bottom*: Variation of the peak current intensity (DPV) of compounds **13** (50 μM), **14** (50 μM), **10** (50 μM), **11** (25 μM) and **12** (12.5 μM) in the presence of increasing amounts of Con A. Each measurement was repeated three times and the standard deviation of the data was always below 6.2%.The dashed lines represent the best least square fit of the experimental data to a model for equal and independent binding sites.

Control experiments were carried out for compounds **10** and **12** using bovine serum albumin (BSA) by ITC and DPV. Negligible variations of the peak currents were observed in

electrochemical experiments upon the addition of 40 □M BSA while ITC titrations gave no exothermic peaks other than the expected heat release due to the dilution process (see Supporting Information). These results indicated that no interaction took place between this protein and such dendrimers.

Sensing Abilities: Once we had demonstrated the protein-induced changes on the current peak intensity of the glycodendrimers **10**-**12** upon binding to Con A, we studied the sensing ability of these compounds through the assessment provided by sensitivity parameters (*P*s). These parameters would enable us to evaluate the extent of the peak current variation induced by the conjugate-protein binding interaction. In our case, we define the sensitivity parameter as $(I_0\text{-}I)/I_0$ where I_0 and I denote the peak current in the absence and the presence of protein, respectively. The *P*s of the glycoconjugates are shown in Figure 6. The order of the sensitivity of the glycodendrimers to Con A is **13** < **14** < **10** < **12** < **11**, showing a clear correlation between *P*s and *K* values. It is important to notice that *P*s and *K* refer to different concepts and therefore they may or may not show the same trend depending on each case. While *K* depicts the stability of the complex, *P*s, as defined above, assesses the peak current decrease induced by the dendrimer-protein binding interaction and is related in part with the number of electrons exchanged by the redox moieties in the presence of protein. The peak current decreases with the increase of the number of electroactive units that incorporates to the low-diffusing cross-linked complex upon the binding with the lectin. That is related with the stability of the complexes, but also with the number of ferrocene units participating in the complex, which depends on the generation of the dendrimer and the stoichiometry of the complex, and with the difference in the diffusion coefficients of the free dendrimer and the complex. However, in our case the binding affinity and thus the "sequestering" of the electroactive units by the protein seems to be dominant factor.

Glycodendrimer **11** having 8 mannose-ferrocene units is the most sensitive of the series for Con A at concentrations of 10 μM. At that concentration Con A is detected with slightly higher sensitivity by dimannosylated monoferrocene **14** than by the monovalent conjugate **13**. Likewise, the sensitivity of tetravalent **10** is 2.5-fold that of monovalent **13** and slightly lower than that of the hexadecavalent **12**. The sensing ability of octavalent glycodendrimer **11** toward Con A at the same concentration is almost 1.5 and 1.4-fold of that of tetravalent **10** and hexadecavalent **12**, respectively. At a higher concentration of the protein (30 μM), dimannosylated monoferrocene **14** is 1.6 times more sensitive than monovalent **13**. At the same concentration, Con A is detected by octavalent Man-Fc **11** with a sensitivity 3.6 and 1.2 times higher than monovalent **13** and tetravalent **10**, respectively, and with a similar sensitivity than hexadecavalent dendrimer **12**.

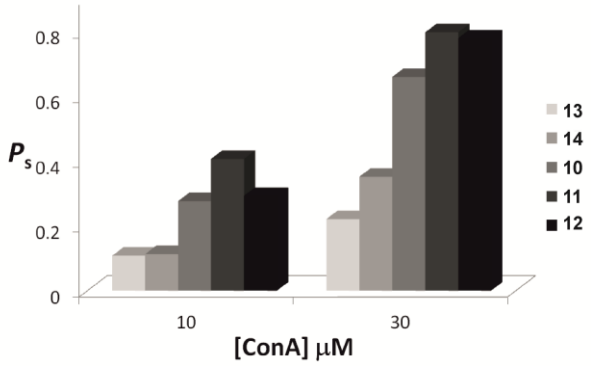


Figure 6. Sensitivity parameters (P_s) of compounds **13** (50 μM), **14** (50 μM), **10** (50 μM), **11** (25 μM) and **12** (12.5 μM) in the presence of different concentrations of Con A (10 and 30 μM). Each measurement was repeated three times and the standard deviation of the data was always below 6.2%.

CONCLUSIONS:

We have synthesized a series of new PAMAM-based dendrimers of generations G0 to G2 having mannopyranosylferrocenyl moieties in the periphery. Such mannosylferrocenylated dendrimers showed, by both calorimetric and electrochemical methods, an enhanced affinity for Con A, as compared with mono- and divalent analogues. This behaviour is attributed to the multivalent effect. In addition, both multivalent binding interaction and multielectron exchange due to the dendritic ferrocene presentation result in an improvement of the redox sensing abilities. Therefore, multiple ferrocene–mannose dendrimers can be used as efficient electrochemical sensors for the detection of Concanavalin A.

ASSOCIATED CONTENT

Supporting Information. Sulfuric acid-phenol assays, ^{1}H and ^{13}C NMR spectra for compounds **2**, **6-8** and **10-12**, mass spectra for compounds **6-8**, **10** and **11**, IR spectra for compounds **6-8** and **10-12**, ConA DPV titrations for compounds **11** and **12-14**, BSA control experiments and ConA ITC titrations for compounds **10** and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Mailing address: Department of Chemistry and Physics, University of Almería, Carretera de Sacramento s/n, 04120 Almería, Spain. Fax: +34-950-015481. Telephone: +34-950-015619. E-mail: avargas@ual.es.

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