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Multivalent Presentation of Mannose on Hyperbranched Polyglycerol and their Interaction with Concanavalin A Lectin

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In this paper we describe the synthesis of multivalent mannose derivatives using hyperbranched polyglycerols (hPG) as scaffold with different linker structures. Grafting of protected mannose (Man) units is achieved via either an anomeric azide or propargyl ether onto complementarily functionalized alkyne or azido polymer surfaces by Cu(I) catalyzed Huisgen click chemistry. NMR, DLS, IR, SEC, and elemental analysis have been used to characterize the hPG-Man compounds. The surface availability and bioactivity of Man modified polymers were evaluated using a competitive surface plasmon resonance (SPR) based binding assay by interactions of the glycopolymers with Concanavalin A (Con A), a lectin that binds mannose containing molecules. Obtained results indicated that the novel glycoarchitectures presented in this work are able to efficiently recognize mannose binding Con A, from the micro to the nanomolar range, while the corresponding monovalent mannoside (methyl-Man) requires millimolar concentrations. The results provide an interesting structure activity relationship for libraries of materials that differ in the linkage of the sugar moiety presented on a biocompatible polyglycerol scaffold.

Introduction

Multivalency is essential in many biological interactions including carbohydrate mediated processes.[1] The low affinity of single ligand-receptor interactions (dissociation constants are in the millimolar concentration range) is compensated by clustering of the receptors and their respective ligands.[2]

Oligosaccharides are important mediators for a large number of biological processes, such as cell-cell recognition and adhesion, bacterial and viral infection, immunological recognition of tumor cells and pathogens, and hormone-cell recognition.[1-3] Therefore, various diseases, and infections can be pharmacologically addressed by the administration of synthetic glycoconjugates which mimic terminal oligosaccharides and can block the recognition, or adhesion processes.[4]

Many proteins that participate in multivalent interactions are oligomers, such as the lectin Con A, a mannose specific lectin, extensively used in model studies with glycopolymers to monitor their binding selectivity. The Con A monomer is a 26 kDa protein with one carbohydrate binding site. In the pH range 5.0 - 5.6, Con A exists as a dimer, while at higher pH of 7.4 Con A dimers associate into tetramers and present four distinct binding sites (~65 Å apart) for mannopyranosides.[5] Different chemical approaches have been developed to study carbohydrate interactions, all of them are based on the multivalent presentation of carbohydrate ligands.

Multivalent interactions have several advantages in comparison to monomeric ones and are often used by nature to control a wide variety of cellular processes, as they bind much more strongly to the interacting protein partner. The binding affinity of carbohydrate-receptor interactions is influenced by a number of properties, e.g. an optimal spacer length and a limited flexibility of the ligands which is necessary to obtain high affinity binding.[6] Another important aspect is the local enrichment of receptors inside the membrane.

The density of binding sites on a multivalent ligand results in a greater number of receptors bound per polymer, faster rate of clustering, and reduced interreceptor distances.[7] The binding mechanism of such multivalent macromolecular ligands is still an area under investigation.[8]

Strong binding enhancements resulting from ligand presentation on different scaffolds have been demonstrated in numerous studies; including glycosylated liposomes,[9] glycoproteins,[10] poly-L-lysine derivatives,[11] and glycopolymers (linear[12] to dendritic[13]) to interpret the mechanistic details of binding processes with lectins. The majority of linear polymeric ligands were copolymers or acrylamide/acrylic esters previously reported by Roy,[14] Whitesides,[2a, 15] and other groups.[16]

Kiessling and coworkers have shown that glycopolymers obtained by ruthenium catalyzed metathesis polymerization (ROMP)[17] can efficiently interact with both soluble and cell-bound lectins. Although the molecular weight distribution of the

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prepared polymers was narrower than for polymers prepared by a radical method, significant polydispersity remained. The dendrimers applied in Con A studies were mainly based on poly(amidoamine) (PAMAM) core, used by Cloninger,[5, 18] Roy,[19] Okada,[20] Lambert[21] and coworkers, which are known to have a strong generation dependent binding affinities.

Following this principle we described a polyvalent system, consisting of a biocompatible polymer core (hyperbranched polyglycerol, hPG) to which mannose residues were covalently linked. The newly formed glycoarchitectures are water soluble, differently linked to the core, and are able to display a large number of mannose on their periphery. In order to provide valuable information about multivalent binding we synthesized hyperbranched polyglycerol cores with different functionalizations, different chain lengths and degrees of carbohydrate shell functionalities. Recently,[22] we reported the synthesis of galactose neoglycopolymers by Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition process. Our approach involved the grafting of sugar azides onto a polyalkyne clickable scaffold that was successfully used for selectin inhibition studies. Currently, hPGs attract attention as they are very promising for biomolecular applications due to their high solubility in water and biocompatibility. The multivalent substrate used here is a neutral, hydrophilic, branched polyether which can be considered comparable to the widely used PEG based materials with regard to toxicity and biocompatibility.[23]

In the present work, hPGs with differently linked terminal Man residues (10, 33, and 60 per hPG) have been prepared and physicochemically characterized for application in Con A binding studies. Glycoconjugate - Con A interactions were evaluated by two different types of binding assays: aggregate formation was monitored by turbidimetric measurements and binding studies by SPR. In the competitive SPR binding assay the ability of hPG-Man to inhibit binding of Con A to a Man functionalized surface was analyzed. SPR is an accurate method to determine binding affinities, and can be applied in addition to other widely used techniques like the inhibition of hemagglutination assay (HAI), isothermal titration microcalorimetry (ITC), and the enzyme linked lectin assay (ELLA).[24]

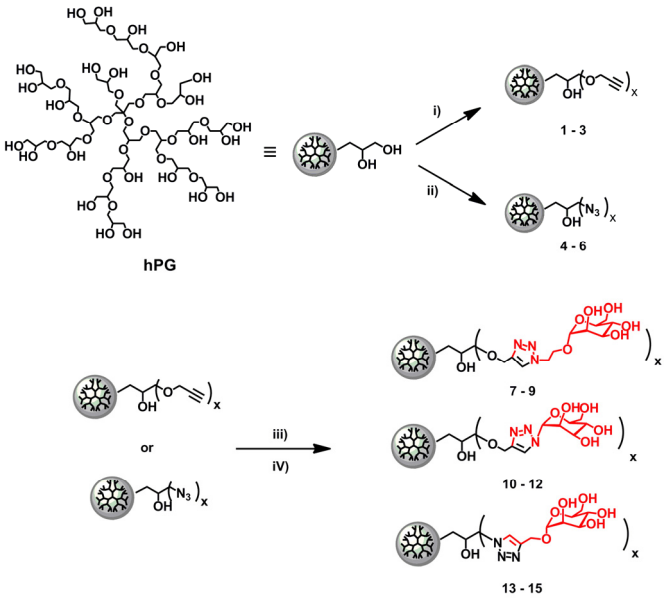
Results and Discussion

Synthesis and characterization of mannose glycopolymers

hPG (M_n= 5 kDa) was prepared according to previously published procedures.[25] hPGs are ideal scaffolds for the multivalent presentation of several functional groups, i.e. carbohydrates, due to their biocompatible properties.[23c, 26] It can be synthesized on a kilogram scale by ring opening polymerization of glycidol[25] and contains linear monohydroxy and terminal dihydroxy functional groups, which are readily functionalizable.[26a] Its high loading capacity (13.5 mmolg^{-1}), low polydispersity (PDI= 1.2), good solubility in a wide range of solvents (depending on the functional groups and degree of functionalization (DF), and chemical stability makes it a promising material for the use as a scaffold for biomedical applications.[26a] The functionalization of hPG and Man derivatives were achieved by employing previously reported experimental conditions. Initially, a fraction of free hydroxyl groups on the hPG core (M_n= 5 kDa) were converted either to propargyl ethers[22] with controlled and varied degrees of functionalization **1**–**3** (15%, 50%, and 90%), or the hydroxyl groups were transformed into azides[27] (**4**–**6**) having same functionalities as mentioned above (Scheme 1).

The corresponding functionalized mannose residues were synthesized using well established procedures. Propargyl α-D-mannopyranoside derivative was obtained from peracetylated α,β-D-mannopyranose by glycosidation with propargyl alcohol and $BF_3{\cdot}OEt_2$ in quantitative yield.[28] The 2-azidoethyl-α-D-mannopyranoside derivative was synthesized by glycosidation reaction,[29] respectively. In case of azido–Man the azido functionality at the anomeric position was introduced via the corresponding peracetylated α,β-D-mannopyranose by reacting with $TMSN_3$ catalyzed by $SnCl_4$.[30] Subsequently, the Man residues were clicked onto the prefunctionalized surface of hPG via triazole linkage by standard Sharpless/Huisgen click chemistry[31] in an overall highly efficient process.

The versatile nature of this reaction has led to a tremendous amount of work, mainly due to the quantitative yields and the possibility of carrying out the synthesis in either organic solvents or water. Scheme 1 shows the general structure of the synthesized substrates used in this study.



Scheme 1. Schematic representation of hPG–Man derivatives (**7**–**15**). The hPG structure is representative and only shows a small fragment of the actual structure (20 mer) for clarity, hPG used was average 5 kDa molecular weight (~66 mer); x= degree of functionalization (DF) 15% (**1**, **4**, **7, 10, 13**), 50% (**2**, **5**, **8, 11, 14**), 90% (**3**, **6**, **9, 12, 15**). i) NaH, propargyl bromide, KI, DMF, 0ºC to rt, overall yield 89%. ii) MsCl, dry Py, NaN_3, DMF, 120ºC, 3h, overall yield 91%. iii) click-chemistry, $CuSO_4$, Na ascorbate, azidoethyl-Man, azido-Man and propargyl-Man, THF:H_2O=1:1, overall yield 85%. iV) NaOMe in MeOH, 12h, rt, quantitative yield.

The synthesized hPG–Man derivatives had different chain lengths, linkages and functionalizations. Compounds **7**–**12** were prepared by attaching the mannoside residues to hPG–alkyne core (**1**–**3**), using the azidoethyl–Man (**7**–**9**) and the anomeric azido–Man (**10**–**12**) carbohydrate units. Alternatively, hPG–Man **13**–**15** possessing the reversed linkage functionality, that is the propargyl group installed on the mannoside residue and the azido group on the hPG scaffold (**4**–**6**), was also similarly prepared to investigate the effect of the aryl triazole ring positioning on binding.

The completion of the click conjugation reaction and the degree of ligand loading (Man) on the hPG were established in all cases by ^{1}H and ^{13}C NMR spectroscopy as well as by size exclusion chromatography (SEC) measurements (see Exp. Section). IR data also confirmed that neither alkyne (3289 cm^{-1}; 2114 cm^{-1}) nor azide (2098 cm^{-1}) residues remain in the final

glycostructures. After deacetylation with NaOMe in MeOH, the unprotected glycoclusters were obtained in quantitative yields. All hPG–Man conjugates were highly soluble in water.

Purification of the compounds was done by dialysis and preparative SEC, respectively. The number-average molecular weight value for each polymer was determined by both ^{1}H NMR spectroscopy and SEC analyses (using linear pullulan standards).

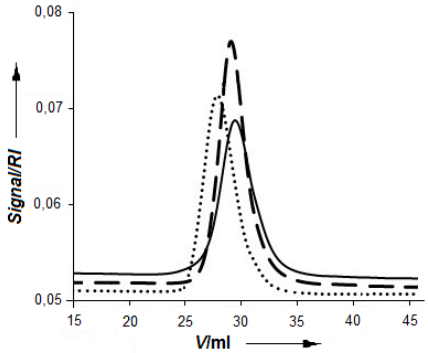


Figure 1. SEC analysis of the deprotected hPG-Man compounds **7** (DF: 15%) with an unbroken line, **8** (DF: 50%) with a dashed line, and **9** (DF: 90%) with a dotted line.

With regard to the size of the hPG–Man derivatives dissolved in water, dynamic light scattering experiments were performed. All the polymeric compounds indicated a size range between 3.5–3.8 nm. Representative ^{1}H and ^{13}C NMR spectra of the conjugates are shown in the supporting information.

Binding Assays

Two different types of binding assays were applied to evaluate the relative binding properties of the above mentioned hPG–Man compounds to Con A. Initially, the rate of Con A precipitation (crosslinking) induced by the polymers **7**–**15** was assessed by a kinetic turbidimetric assay.[28b] Commercially available Con A (Sigma-Aldrich, Germany) was treated with each of the polymers in microtiter plates. Each of the compounds and Con A were tested at a concentration of 0.3 mgml^{-1}. The polysaccharide yeast mannan served as positive control. The raw data are shown in Figure 2. When glycopolymers **7**–**15** were mixed with Con A, insoluble crosslinked complexes could be clearly and readily visualized within 10 minutes of mixing. At physiological pH, Con A exists as tetramer that may assist the formation of stable, insoluble complexes with mannosylated polymers.

The aggregation was monitored by measuring the optical density (O.D.) at a wavelength of 490 nm. The turbidity change of Man functionalized hPGs by the addition of Con A was dependent on the DF value of hPG–Man derivatives. Polymers that displayed the greatest number of binding sites generated the largest clusters. For example, the addition of Con A to the dispersion of hPG–Man **9** and **12** (DF: 90%, ~60 Man units) induced a gradual increase in turbidity and then reached a plateau value almost at O.D.~ 0.6. On the other hand, the increase in turbidity was relatively much smaller (O.D.< 0.3) by using hPG–Man **7** and **10** (DF: 15%). Compounds **13**–**15** were significantly less potent than the rest of the compounds with the same DF. Furthermore, polysaccharide yeast mannan, used as positive control, showed a moderate turbidity change (O.D.= 0.2).

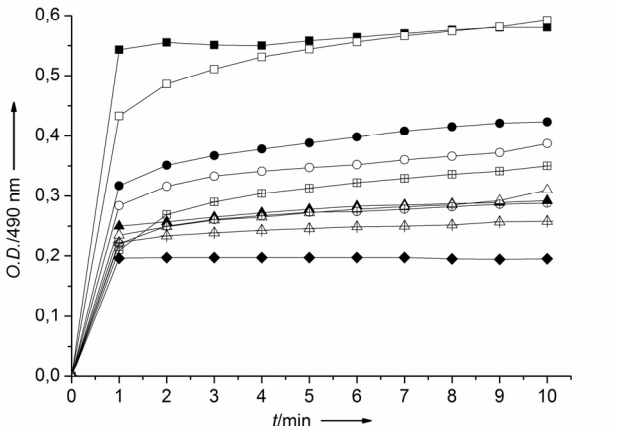


Figure 2. Turbidimetric analysis at 25ºC. Crosslinking of the compounds with Con A after several minutes was observed. The Con A was treated with 0.3 mgml^{-1} of yeast mannan positive control (♦), **7** (▲), **8** (●), **9** (■), **10** (△), **11** (○), **12** (□), **13** (◬), **14** (⌖), and **15** (⌗). Measurements were done in PBS using an ELISA plate reader and were the average of triplicate values.

The turbidimetric analysis described above clearly demonstrated the ability of hPG–Man derivatives to precipitate lectin in solution. It is very clear that there was a steep increase in the rate of turbidity change at a high content of mannose-carrying hPGs (**9** and **12**). The best derivative was compound **9**, because of an almost quantitative precipitation of the tetrameric lectin occurring after only 1 minute (O.D.= 0.543).

Secondly, a competitive SPR based binding assay has been performed to evaluate selected hPG–Man derivatives as inhibitors of Con A. *α*-D-Mannose (Man) coupled on a polyacrylamide (PAA) backbone was immobilized on the surface of a sensor chip, while Con A was injected in a continuous flow over the surface (Figure 3).

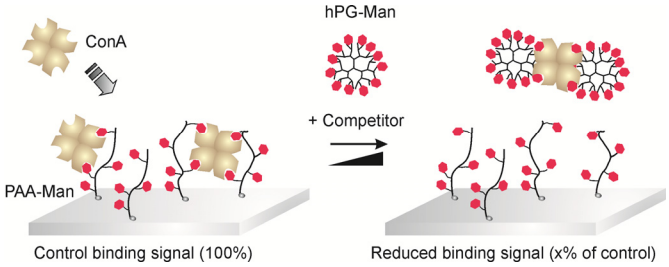


Figure 3. SPR assay design. The biosensor strategy was to measure binding of 500 nM tetravalent Con A to surface immobilized mannose, presented on a polyacrylamide backbone (PAA–Man). The addition of an increasing concentration of soluble mannose functionalized hPG competitors (hPG–Man) resulted in shielding of Con A binding to the surface bound mannose residues. Consequently a reduced binding signal was recorded.

The binding measurements were approved at a fixed concentration of Con A in the presence of the hPG–Man conjugates **7**–**15** at varying stoichiometric ratios. Binding of 500 nM Con A to immobilized Man without the presence of any compound was set as 100% binding. Subsequently, the binding of 500 nM Con A was measured after incubation with different compound concentrations (Figure 4).

These Man functionalized hPGs **7**–**15** (10, 33, and 60 terminal Man per molecule) showed strong inhibition of Con A binding with IC_{50} values in the micro- to nanomolar range. Hyperbranched polyglycerol itself did not exhibit any nonspecific binding to Con A, suggesting that Man units are entirely

responsible for the inhibition. The results for Con A lectin inhibition of binding to immobilized mannosylated polyacrylamide by the hPG–Man derivatives are reported in Table 1 (data are shown for compounds **7**–**9**). The Man multimer **7** (DF: 15%, 10 terminal Man units) showed binding in the micromolar range (7000 nM), which represents an affinity enhancement of up to 107-fold over the monovalent methyl-Man (Me-Man) counterpart. Furthermore there was also a marked increase in inhibitory potencies arising from glycopolymers **8** (DF: 50%, 33 terminal Man units) and **9** (DF: 90%, 60 terminal Man units), with IC_{50} values of 280 and 35 nM, respectively.

Table 1. SPR inhibition assay results[a]

Compound[b]	No. Man residues[c]	IC_{50} [nM] of dendritic polymer	IC_{50} [nM] per mannose unit[d]	Relative activity/ Man
Me-Man	1	-	750,000	1
7	10	7,000	70,000	11
8	33	280	9,240	81
9	60	35	2,100	357

[a] Each reported value represents at least three assays; data are shown for compounds **7**–**9**. [b] hPG: average molecular weight 5 kDa was used; M_w/M_n= 1.2; DB (degree of branching)= 0.57. [c] Determined by ^{1}H NMR based on integration of Man-H, and by IR. [d] Values represent IC_{50} values in nanomolar concentrations based on mannose sugar unit concentration.

The data from these experiments revealed that the number of lectin tetramers bound per polymer increased with increasing epitope density and that the hPG–Man prepared in this work acted as multivalent ligands. When expressed on a per Man basis, all of the glycopolymers showed an increase in potency (11-, 81-, 357-fold for compounds **7**, **8**, and **9**). This enhancement in activity, compared to the corresponding monovalent ligand on a per mole of Man basis, was noted by Lee and coworkers in 1995 and is often referred to as the "glycoside cluster effect."[8a, 24a, 32] Multivalency of ligands is an important element for high affinity binding of Con A. In both binding assays we applied, the same ranking in affinity occured within each compound with respect to the number of mannose residues. By increasing the degree of functionalization, the binding affinity was amplified. Highest binding affinity was found in the SPR measurements as well as in the turbidity assay for compound **9**.

Effect of the distance between the hPG and Man residues on the recognition by Con A

Spacer length, separating the sugar from the polyglycerol core appeared to be crucial in controlling the interaction of the compounds with the lectin in solution. The derivatives having the longer spacer (**7**–**9**), interacted more significantly with Con A than compounds devoid of any spacer (**10**–**12**). Compounds **13**–**15**, which have a single methylene and mannoside moiety attached to the triazole ring, showed less potency in binding to Con A. There are many reports that hydrophobic aglycon promotes the recognition of sugars by lectin.[11a, 33] This could be a reason for a favorable change in entropy or favorable interactions within the binding site of Con A.

Previous reports by Brewer suggested that the binding of multivalent carbohydrates to Con A arises from increasing positive entropy ($T\triangle S$) contributions relative to monovalent analogs. The enthalpy for binding, $\triangle H$, was shown to be directly proportional to the number of binding epitopes.[34] Higher sugar loading onto hPG therefore promoted higher binding activity.

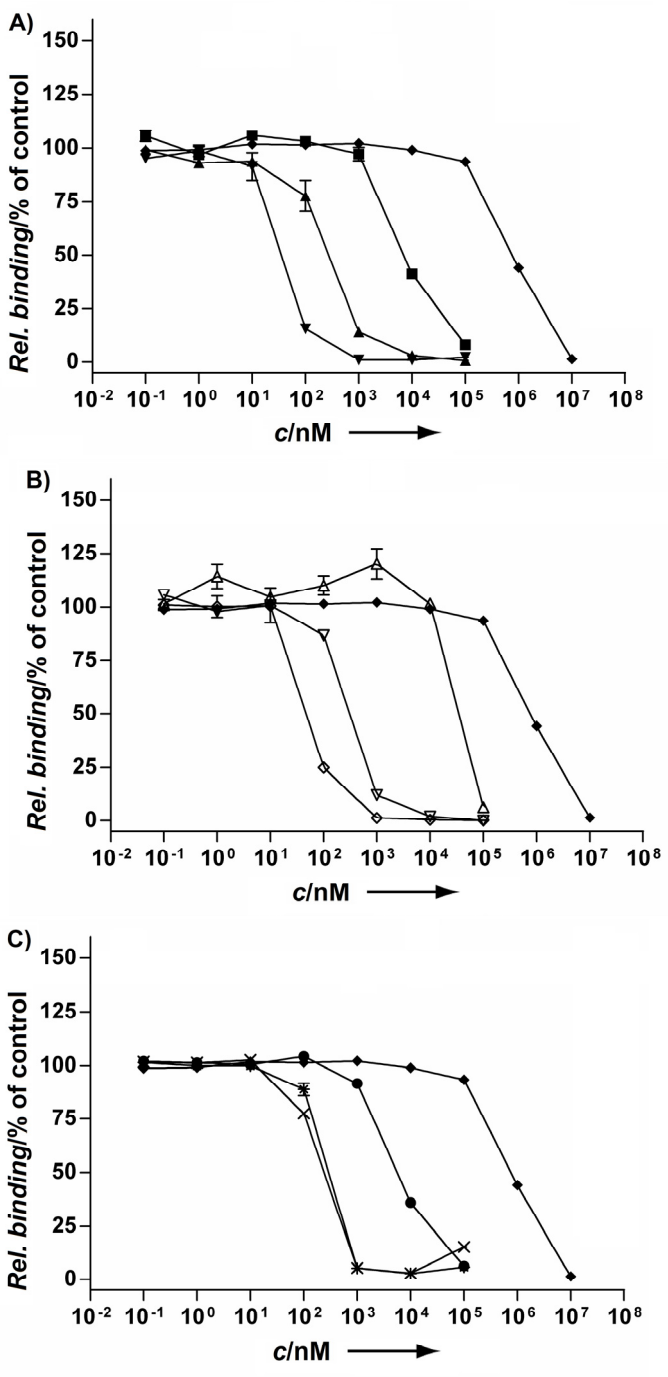


Figure 4. Binding curves to Con A. Percent binding (y axis) is plotted against the concentration of the soluble polymeric compounds (x axis). The Con A was preincubated with different concentrations of A) methyl-Man (♦), **7** (■), **8** (▲), **9** (▼); B) methyl-Man (♦), **10** (△), **11** (▽), **12** (□); C) methyl-Man (♦), **13** (●), **14** (✳), **15** (×). Each data point represents the mean (±SEM) of at least three measurements.

Determination of Con A binding sites on Man-hPG

To gain insight into the stoichiometry of Con A binding per functionalized hPG we performed a precipitation assay (Figure 5), initially described by Brewer.[10c] From this assay we calculated the number of Con A molecules bound per hPG at the saturation limit. It turned out that compound **7** (10 Man) binds 0.9 Con A, while compounds **8** (33 Man) and **9** (60 Man) both bind 2.9 Con A

molecules. The unfunctionalized hPG has only a molecular weight of 5 kDa, therefore we expected for steric reasons that only a few tetrameric Con A (104 kDa) proteins are able to bind simultaneously. The approximately 1:1 binding mode to the low functionalized compound **7** is therefore not surprising. Obviously, by increasing the ligand density up to 3 Con A molecules are able to address the functionalized hPG (compounds **8** and **9**). While we see differences in both binding assays with respect to the ligand density, in the precipitation assay compound **8** and **9** behave the same. An increase from 33 Man to 60 Man ligands does not result in additional Con A binding, probably because of steric hindrance. We explain this difference with varying incubation times of the three assays. Both the SPR based and the turbidity assay are performed within minutes, in this precipitation assay the incubation time was 12 hours, sufficient time to rearrange and optimise receptor-ligand positioning.

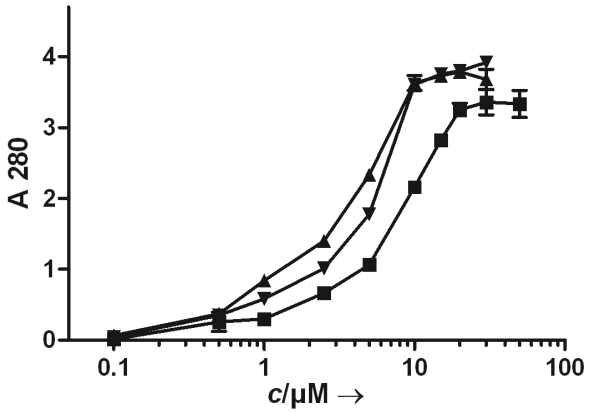


Figure 5. Quantitative precipitation of Con A. The assay was performed with increasing concentrations of hPG-Man derivatives: compound **7** (■), **8** (▲), **9** (▼) to determine Con A binding to hPG-Man. Stoichiometry of binding was calculated from the polymer concentration (x axis) necessary to quantitatively precipitate Con A. Redissolved protein concentrations are shown as A_{280} (y axis). Each data point represents the mean (±SEM) of at least three measurements.

Conclusion

In summary, we have synthesized well defined multivalent hPG–Man conjugates which display a large number of Man units on their periphery (10 up to 60 Man). Our general strategy here involved the conjugation of appropriate azido or propargylated Man residues to the prefunctionalized hPG surfaces bearing terminal alkyne or azido groups, and afforded glycopolymers **7**–**15**. This strategy is efficient for the synthesis of libraries of materials that differ in the nature of the carbohydrate moiety, presented on a well defined polymer scaffold.

The generated hPG–Man derivatives have been tested for Con A binding by quantitative precipitation in a turbidimetric assay and by SPR measurements for competitive binding. The mannose-carrying polyglycerols were recognized by Con A, which was proven by the increase in turbidity of the polymeric suspension after mixing with Con A. SPR experiments clearly demonstrated as well, that highly functionalized hPG–Man efficiently inhibited Con A/Man interactions and their potencies on a per Man basis are higher than those obtained for monovalent methyl-Man. Results showed that the lectin binding to ligands on hPG is profoundly affected by how the ligands are displayed on their surface. By increasing the level of Man substitution the performance of the dendritic competitor was improved. Values for compound **13**–**15** are much lower, they had less activity. Binding affinity for the multivalent ligands observed in the turbidimetry assay, were qualitative similar to those obtained via SPR. The most potent multivalent derivatives were compounds with the highest functionalization of Man residues (**9** and **12**, 60 Man)
The maximum stoichiometrie of Con A binding to hPG-Man binding is 3:1, but it seems possible that this binding mode can be achieved at an even lower number of 33 Man (compound **8**). To further improve binding affinity, not a higher functionalization, but an increase of the hPG core should be appropriate, to provide more space for protein binding. Further research will address targeting of human mannose-receptors in cell-culture experiments. Initial toxicity studies on the mouse fibroblast cell line NIH-3T3 gave us hints for adequate working concentrations (Figure S1, Supporting information). Concentrations of compounds **7**–**9** up to 10^{-5} M showed no toxicity compared to the unfunctionalized control, but at a tenfold increase functionalization became toxic for so far unknown reasons

The results of this study have allowed us to identify the best inhibitor among these hPG derivatives and have confirmed the strong multivalency effect for potent inhibition. In contrast to the high affinity binding of multivalent hPG–Man derivatives towards Con A, monovalent methyl-Man just bound Con A in the millimolar range. Even when expressed on a per monosaccharide basis, the relative inhibitory potencies of the hPG–Man derivatives were still high (up to 357 times more potent) than the methyl-Man.

Experimental Section

Surface Plasmon Resonance (SPR) measurements
Instrumentation: Experiments were performed on a Biacore X instrument (Biacore AB, Uppsala, Sweden) at 25°C. Con A was obtained from Calbiochem/Merck KGaA, Darmstadt, Germany. The ligand immobilization involved the use of HBS–EP buffer (Biacore AB), consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0,005% Surfactant P 20. Running buffer during the assay was 10 mM HEPES, pH 8.5, with 100 µM $CaCl_2$. The ligand immobilization procedure was performed similarly as previously described.[35]

Biotinylated α-*D*-mannose-PAA (20 mol-%) (Lectinity Holdings, Moscow, Russia) was immobilized on a sensor chip SA (Biacore AB). For reference purposes, biotinylated N-acetyllactosamine-PAA (LacNAc-PAA) (Lectinity) was immobilized on a second lane of the same chip. The final concentration of each conjugate during injection was 4.2 µg/ml. For better performance, the sensor chip was initially conditioned with three consecutive 1 min injections of 1 M NaCl in 50 mM NaOH before starting immobilization. Before loading, each sample was incubated with the compounds for 18 min at room temperature at final inhibitor concentrations of 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM. The samples were injected over the reference and α-D-mannose-PAA lane at a flow rate of 30 µl/min. Each cycle consisted of a 70 sec period of sample injection (association phase), a 180 sec undisturbed dissociation phase, and a wash of the flow system with 100 mM glycine–HCl, pH 2.5. The chip was loaded with each conjugate up to a baseline shift of 800 resonance units (RU).

The immobilization procedure was followed by several washes with running buffer to equilibrate the chip surface. For data evaluation the reference lane data were subtracted from α-*D*-mannose-PAA lane data. Responses of the sample injections were extracted between report points set at the start of the injection (0 sec) and at the end of the dissociation phase (250 sec). The final response values were used for curve creation. Each point represented the mean value (SEM) of at least 3 measurements.

Turbidimetric analysis

Turbidimetry experiments were performed in microtitration plates where 100 ml/well of stock Con A solution, prepared from 0.3 mgml^{-1} PBS, was mixed with 100 ml/well of a mannosylated hPG solution and incubated at room temperature for 12 min. The turbidity of the solutions was monitored by reading the optical density at 490 nm at regular time intervals until no noticeable changes could be observed. Each test was done in triplicate.

Stoichiometry of Con A binding

A quantitative precipitation assay was performed to determine the stoichiometry of Con A binding to compounds **7**–**9** as described previously[10c, 18a] with minor modifications. Various concentrations of glycodendrimers **7**–**9** were incubated with a constant amount of Con A (final concentration 33 μM) in a 50 μl reaction volume. The reaction was performed at 25°C in 0.1 M Tris/HCl buffer, pH 7.2 with 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Mixtures were incubated for 12 h and subsequently centrifuged at 5000 rpm for 5 min to pellet the precipitate. The pellet was washed three times with 50 μl of cold buffer and then dissolved in 50 μl of 0.1 M methyl mannoside. The concentration of released Con A in each solution was determined by UV absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer (peqlab, Germany). The values given are the average of three independent measurements. The ratio of Con A per dendrimer was calculated from the molar concentration of the compounds when maximum precipitation starts.

Materials and Methods

Unless otherwise stated, all starting materials were obtained from commercial suppliers and used without further purification. Reactions requiring dry or oxygen-free conditions were carried out in dried Schlenk glassware under argon.

^{1}H and proton decoupled carbon NMR spectra were recorded on ECX 400 (400 MHz and 100 MHz for ^{1}H and ^{13}C, respectively) spectrometers at ambient temperature. Chemical shifts δ are given in parts per million (ppm) according to the literature. The spectra were calibrated using the deuterated solvent peak. Flash column and thin layer chromatography were performed with Merck Silica Gel 60. Reactions were monitored by thin layer chromatography (TLC) on a precoated plates of silica gel 60, F_{254} and were visualized under ultraviolet light or via additional staining (20% ethanolic sulfuric acid), followed by heating.

Dialysis (benzoylated cellulose dialyse tubes, Sigma–Aldrich, width: 32 mm molecular weight cut–off (MWCO= 1000, 8000 gmol^{-1}), was performed in a 1 L beaker, changing the solvent 4 times over a period of 24 hours. Ultrafiltration was done under 5 bar N_2, by using cellulose membranes (MWCO= 5000, 10000 gmol^{-1}). Freeze drying was done by Christ Alpha 12 LD lyophilizator. Elemental analyses were performed on a Perkin-Elmer EA 240. Infrared spectra (IR) were recorded on a Nicolet 5 SXC FT–IR spectrometer (operating from 4000–400 cm^{-1}) on KBr pellets. Dynamic light scattering (DLS) measurements were conducted using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). Aqueous samples were filtered through 0.2 μm filters prior to analysis.

The polymer molecular weigths and polydispersities (M_w/M_n; M_w: weigth-average molecular weight, M_n: number-average molecular weight) were determined on analytical size-exclusion chromatography (SEC, PSS Agilent 1100 system) equipped with three Suprema (10 μm) columns (3×ID8.0×300 mm) and a refractive index detector at 25ºC. The elution phase was water with 0.05% NaN_3 and the system was calibrated by narrow polyethylene glycol (M_w range: 106–4×10^5 gmol^{-1}) or pullulan standards (M_w range: 1080–6.41×10^5 gmol^{-1}) using a PSS Win-SEC software.

Synthetic Procedures

hPG–propargylether (**1**–**3**). hPG of average molecular weight 5 kDa, with conversion of the OH groups to propargyl ether (degree of functionalization: DF: 15%, 50% and 90%) was achieved quantitatively according to our previously published procedure.[22] The conversion of hydroxyl groups into the alkyne functionality was confirmed by the appearance of a medium and a weak alkyne C–H stretching band in the IR spectrum at 3288 and 2113 cm^{-1}, respectively. All functionalities had similar NMR spectra except for the change in peak intensity (intensity was higher as DF increased).

Compound ***3***. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 4.31 (s, sec. O$\underline{CH_2}$C≡CH), 4.11 (s, primary O$\underline{CH_2}$C≡CH), 3.86–3.43 (m, hPG backbone), 2.41 (s, C≡C$\underline{H}$), 1.42 (m, C$\underline{H_2}$-hPG starter unit), 0.81 (t, C$\underline{H_3}$-hPG starter unit); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, δ): 81.5–56.3 (hPG backbone and $\underline{C}$≡CH), 30.1 (C≡$\underline{C}$H); IR (KBr): ν(cm^{-1}) = 3440 (ν-H_2O), 3288 (m, ν-C≡CH), 2920, 2880 (s, ν-CH_3, ν-CH_2), 2113 (w, ν-C≡CH). $M_{w,NMR}$= 7280 Da, $M_{n,SEC}$= 6700 Da, M_w/M_n= 1.13.

hPG–azide (**4**–**6**). For their synthesis a two-step modification procedure was used to convert the hydroxyl groups of hPG into mesyl groups, followed by nucleophilic substitution with NaN_3, turning mesyl into azide functionalities.[27] The synthesized functionalities were 15%, 50%, and 90%. All 3 functionalities had similar NMR spectra except for the change in peak intensity (intensity was higher as DF increased). *Polyglycerolazide* **6** (DF= 90%): Polyglycerol (2 g, 27 mmol OH–groups) was dissolved in dry pyridine (16 ml). The solution was cooled down to 0ºC and a solution of MsCl (1.2 equiv, 2.5 ml, 33 mmol, 3.7 g) dissolved in small amount of pyridine was added dropwise. Reaction was carried out under inert gas atmosphere and exclusion of water. The resultant brown mixture was stirred for 16 h in thawing ice bath. Then the reaction was quenched with ice and the resultant precipitation, after decantation of the liquid, was washed with water and dialysed in acetone to give a light brown honey like product. Conversion was quantitative, yield 91%. The obtained mesylated polyglycerol (3.5 g, 21.8 mmol OMs groups) were dissolved in DMF (45 ml). After addition of NaN_3 (5 equiv, 109 mmol, 7 g) the mixture was heated at 120ºC for 4 h. After cooling, the excess of NaN_3 was filtered off through Celite and DMF was removed via cryo–distillation. Following extraction with $CHCl_3$ and water, the residue was further purified by dialysis (MWCO: 1 kDa) in $CHCl_3$. Conversion was quantitative, yield 87%. ^{1}H NMR (500 MHz, $CDCl_3$, δ) = 3.96–3.21 (m, hPG backbone), 1.83, 0.85 (hPG- starter); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, δ): 81.9–66.3 (hPG backbone), 60.5 (sec. functionalized groups), 51.3 (primary functionalized groups); IR (KBr): ν(cm^{-1}) = 3405 (s, ν-OH), 2922–2876 (m, ν-CH_2), 2102 (s, ν_{as}-N_3.). $M_{w,NMR}$= 6500 Da, $M_{n,SEC}$= 6310 Da, M_w/M_n= 1.10. Degree of functionalization was determined as well by elemental analysis: N calcd: 38.76%; N found: 37.34%.

General synthesis of hPG–Man (**7–15**). The final acetylated Man polymers were obtained via click chemistry. To a solution of THF/H_2O (1:1) propargyl/azido–hPG (1 equiv) and the complementary azido/propargylated–Man (1.5–2 equiv) were added in the presence of Na ascorbate (10–20 mol-%), followed by the catalyst $CuSO_4{\cdot}5H_2O$ (5–10 mol-%). The mixture was stirred vigorously for 16 h at room temperature and was monitored by IR measurements. After completion of the reaction, the mixture was extracted with dichloromethane, washed with EDTA sat. aq. solution, water and brine. After drying over $MgSO_4$, solvent was removed in vacuo and the residue was purified by dialysis/ultrafiltration in acetone (for high functionalized products) or in methanol (for low functionalized products). The acetylated products were unprotected using NaOMe in MeOH. Final products were dialysed in distilled water for 24 h and lyophilized, which provided the unprotected macromolecules in quantitative yields. The purity of the products was determined by NMR, IR and SEC analysis. After clicking, deprotection and purification steps the characteristic bands for alkyne and azido groups disappeared, while a broad signal at. 3400 cm^{-1} corresponding to the sugar hydroxyl groups became evident. All hPG–Man conjugates were highly soluble in water and all functionalities had similar NMR spectra except for the change in peak intensity (intensity was higher

as DF increased). The Man density on polymer (degree of functionalization) was analyzed from the intensity of the anomeric protons by ^{1}H NMR integration: Man–1-H at 6.10 ppm (in azido Man); and at 4.64 ppm (in azidoethlyMan), which were in accordance with the Man ratios employed in their synthesis.

*Compound **9** protected* (DF: 90%). Reaction conditions and workup were as described above. **3** (100 mg, 0.82 mmol) and azidoethyl–Man (1.5 equiv., 516 mg, 1.24 mmol) were dissolved in THF:H_2O (5 ml). To this reaction mixture Na ascorbate (32 mg, 0.16 mmol) and $CuSO_4{\cdot}5H_2O$ (21 mg, 0.083 mmol) were added. The obtained yield was 80% (355 mg). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.76 (s, 60H, C=CH), 5.17–5.10 (m, 180H, Man: H–1, H–3, H–2), 4.80–4.45 (m, Man: H–4, H–6a, H–6b, H–5), 4.15–3.20 (m, Man: -CH_2CH_2- and hPG backbone), 2.10–1.91 (m, 720H, OAcs), 1.18, 0.75 (hPG starter units); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, δ): 170.4, 169.9, 169.6, 169.2 (O=C), 145.5, 144.9 (C=CH), 124.2 (C=CH), 97.5 (C–1, Man), 78.9, 77.6, 71.8, 71.6, 70.7, 69.4 (Man, OCH_2), 69.2, 68.9, 66.3, 65.7, 64.5, 63.6, 62.2, 53.9, 49.6 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6, –CH_2N–), 20.8–20.5 (OAcs, Man); IR (KBr): ν(cm^{-1})= 3430 (ν-H_2O), 2921 (ν-CH_3, ν-CH_2), no peak at 3288 and 2114 (ν-C≡CH), 2104 (ν-N_3).

*Compound **9** deprotected* (DF: 90%). The obtained yield was 77% (233 mg); ^{1}H NMR (400 MHz, D_2O; δ) = 8.08 (s, 58H, C=CH), 4.64 (s, 240H), 4.20–3.45 (m, Man: H–6,-CH_2CH_2-, hPG backbone); 3.08 (s, 60H); ^{13}C{^{1}H} NMR (125 MHz, D_2O, δ): 143.6, 143.3 (C=CH), 124.7 (C=CH), 98.8 (C–1, Man), 77.3, 76.5, 76.2, 72.4, 72.1, 71.4, 70.3, 69.7, 69.2, 68.4, 65.7, 64.9, 64.7, 63.0, 62.7, 62.3, 61.8, 61.5, 59.9, 49.3 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6, -CH_2CH_2-); $M_{w,NMR}$= 22,200 Da, $M_{n,SEC}$= 19,000 Da, M_w/M_n= 1.3. Degree of functionalization was determined as well by elemental analysis: N calcd: 11.35%; N found: 10.87%. The size of **3**, determined by DLS, was 3.8±0.13.

*Compound **12** protected* (DF: 90%). Reaction conditions and workup were as described above. **3** (100 mg, 0.82 mmol) and azido–Man (1.5 equiv., 461 mg, 1.24 mmol) were dissolved in THF:H_2O (5 ml). To this reaction mixture Na ascorbate (32 mg, 0.16 mmol) and $CuSO_4{\cdot}5H_2O$ (21 mg, 0.083 mmol) were added. The obtained yield was 94% (383 mg). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.88 (s, C=CH), 6.10–5.75 (m, 120H, Man: H–3, H–4), 5.32 (s, 60H, H–1, Man), 4.80–3.20 (m, Man: H–2, H–6a, H–6b, H–5, and hPG backbone), 2.10–1.92 (m, 720H, OAcs), 1.25, 0.75 (hPG starter units); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, δ): 170.3, 169.7, 169.5, 169.2 (O=C), 145.8, 145.3 (C=CH), 123.5 (C=CH), 83.7 (C–1, Man), 77.4, 77.2, 71.8, 71.6, 70.6, 68.9, 68.3, 65.8, 65.5, 64.5, 63.6, 62.1, 61.6, 61.4 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6), 20.5–20.4 (OAcs, Man); IR (KBr): ν(cm^{-1})= 3430 (ν-H_2O), 2921 (ν-CH_3, ν-CH_2), no peak at 3288 and 2114 (ν-C≡CH), 2104 (ν-N_3).

*Compound **12** deprotected* (DF: 90%). The obtained yield was 91% (244 mg); ^{1}H NMR (400 MHz, D_2O; δ) = 8.21 (s, 56H, C=CH), 6.10 (s, 60H, H–1), 4.20–3.25 (m, Man: H–2, H–3, H–4, H–5, H–6, hPG backbone); ^{13}C{^{1}H} NMR (125 MHz, D_2O, δ): 144.2 (C=CH), 124.3 (C=CH), 86.3 (C–1, Man), 79.4, 78.1, 76.9, 75.8, 72.8, 72.2, 71.7, 70.8, 69.9, 69.2, 68.7, 68.4, 67.8, 66.5, 66.1, 63.5, 62.9, 62.6, 62.2, 61.8, 60.3 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6); $M_{w,NMR}$= 19,500 Da, $M_{n,SEC}$= 17,419 Da, M_w/M_n=1.24. Degree of functionalization was determined as well by elemental analysis: N calcd: 12.86%; N found: 12.49%. The particle size of **6** was 3.5±0.04.

*Compound **15** protected* (DF: 90%). Reaction conditions and workup were as described above. **6** (100 mg, 0.93 mmol) and propargyl–Man (1.5 equiv., 535 mg, 1.39 mmol) were dissolved in THF:H_2O (5 ml). To this reaction mixture Na ascorbate (32 mg, 0.16 mmol) and $CuSO_4{\cdot}5H_2O$ (21 mg, 0.083 mmol) were added. The obtained yield was 95% (433 mg). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.78 (s, C=CH), 5.25 (m, 240H, Man: H–1, H–2, H–3, H–4), 4.75–3.33 (m, Man: H–5, H–6, –OCH_2, and hPG backbone), 2.16–1.99 (m, 720H, OAcs), 1.25, 0.75 (hPG starter units); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, δ): 170.7, 170.0, 169.9, 169.8 (O=C), 141.8 (C=CH), 123.5 (C=CH), 96.3 (C–1, Man), 77.4, 77.2, 71.8, 71.6, 70.6, 69.4, 69.0, 68.9, 68.3, 66.0, 65.8, 65.5, 64.5, 63.6, 62.4, 62.1, 61.6, 61.4, 55.02 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6), 20.9–20.7 (OAcs, Man); IR (KBr): ν(cm^{-1})= 3430 (ν-H_2O), 2921 (ν-CH_3, ν-CH_2), no peak at 3288 and 2114 (ν-C≡CH), 2104 (ν-N_3).

*Compound **15** deprotected* (DF: 90%). The obtained yield was 88% (252 mg); ^{1}H NMR (400 MHz, D_2O; δ) = 7.75 (s, 54H, C=CH), 4.86–3.35 (m, Man: H–1, H–2, H–3, H–4, H–5, H–6, –OCH_2, hPG backbone); ^{13}C{^{1}H} NMR (125 MHz, D_2O, δ): 142.9 (C=CH), 124.8 (C=CH), 98.6 (C–1, Man), 78.1, 76.9, 76.3, 72.8, 72.2, 71.3, 70.6, 69.7, 69.3, 65.9, 60.2, 58.8, 58.5 (hPG backbone, Man: C–5, C–2, C–3, C–4, C–6, –OCH_2); $M_{w,NMR}$= 19,590 Da, $M_{n,SEC}$= 15,660 Da, M_w/M_n= 1.36. Degree of functionalization was determined as well by elemental analysis: N calcd: 12.86%; N found: 12.53%. The size of **9** by DLS was 3.4±0.11.

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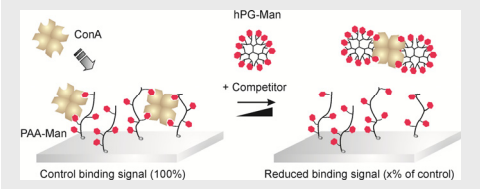
Multivalent dendritic glycoconjugates with high binding affinities for Con A are efficiently prepared and a detailed structure activity relationship revealed the best linker and the degree of functionalization for the Man-polymer conjugates.



*I. Papp, T. C. Shiao, S. Enders, J. Dernedde, R. Roy, R. Haag**

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Multivalent Presentation of Mannose on Hyperbranched Polyglycerol and their Interaction with Concanavalin A Lectin

Supporting Information

Cytotoxicity assay. For the analyses of the toxicity of hPG-Man derivatives 10,000 NIH 3T3 cells (Swiss mouse embryo; fibroblast) were used per well in 96-well plates. The cells were incubated for 4 h (37ºC, 5% CO_2) before hPG-OH and hPG-ManOH (compounds **7**, **8**, **9**) were added (final volume: 200 µl) at different concentrations (0 nM, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M). Samples were incubated for 48h (37°C, 5% CO_2) and relative cell survival was analyzed by MTT assay. 30 µl MTT [5 mg/ml] was added directly to the cells. After 2 h of incubation (37°C, 5% CO_2) MTT was removed and 50 µl formazan solubilizer (205 ml isopropanol, 25 ml, 10% SDS, 20 ml 1 M HCl) was added and incubated for 15 min at room temperature. Absorbance was measured at λ_1 = 570 nm and λ_2 = 630 nm and the amount of untreated cells was set to 100%. Wells without cells were used as blank.

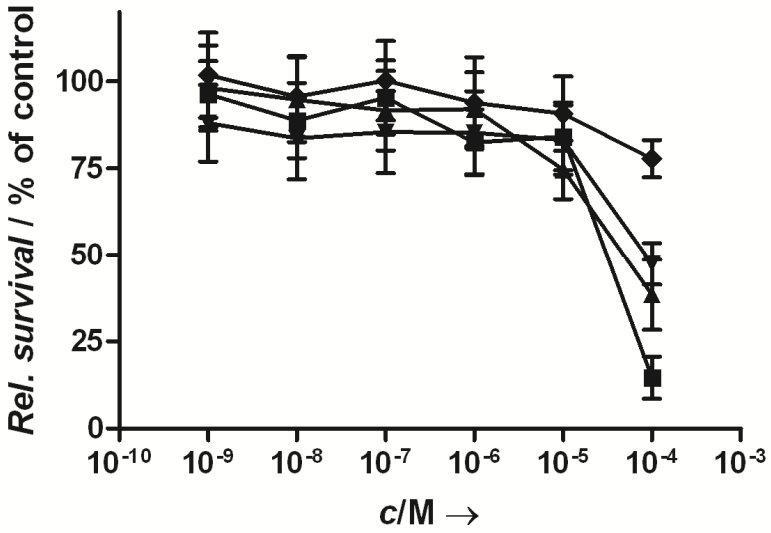
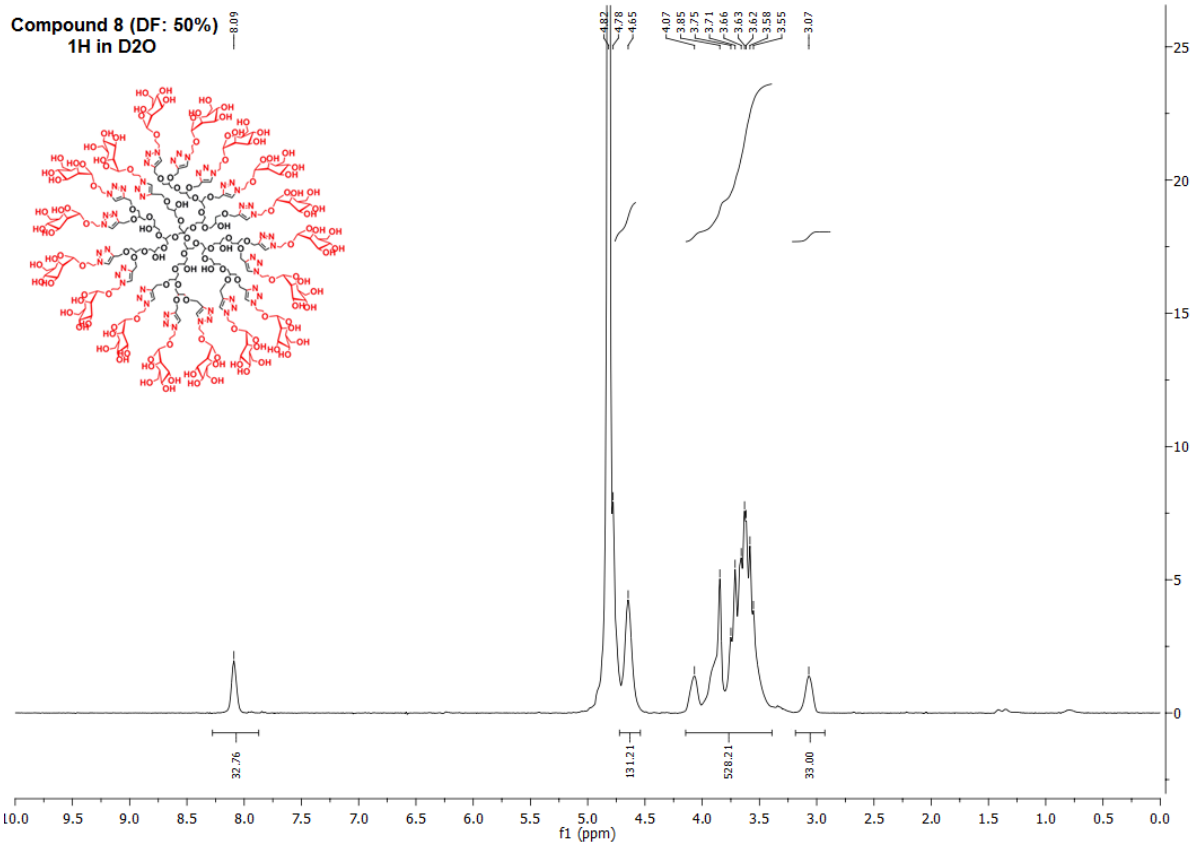
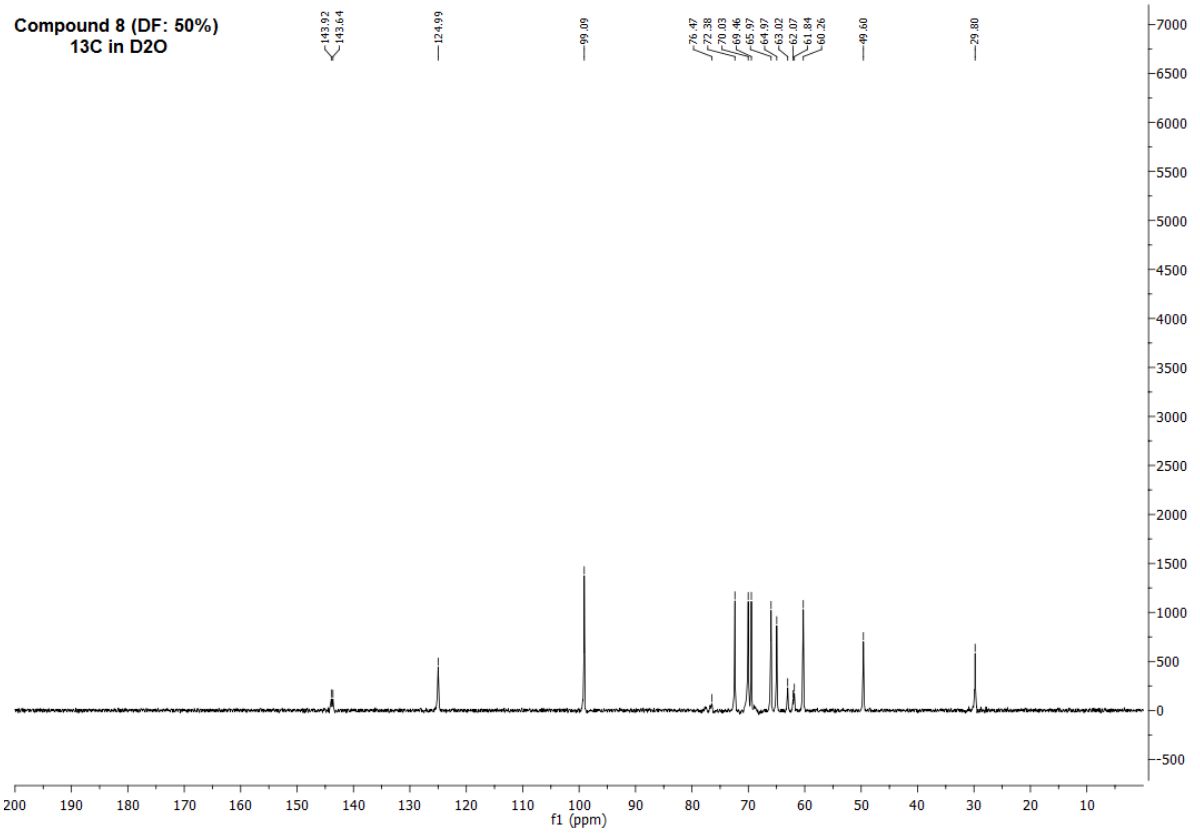


Figure S1. Cell toxicity assay of hPG-ManOH and hPG-OH. NIH-3T3 cells were grown in 96-well plates and incubated in the presence of hPG derivatives for 48h (37°C, 5% CO_2). The relative survival (y axis) is plotted against different concentration (x axis) of the polymeric compounds [hPG-OH (◆) and hPG-ManOH: **7** (■), **8** (▲), **9** (▼)]. Data are presented as mean ± SEM of three independent experiments in triplicates.


Compound 8 (DF: 50%)
1H in D2O
8.09
4.82
4.78
4.65
4.07
3.85
3.75
3.71
3.66
3.63
3.62
3.58
3.55
3.07
32.76
131.21
528.21
33.00
f1 (ppm)


Compound 8 (DF: 50%)
13C in D2O
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143.64
124.99
99.09
76.47
72.38
70.03
69.46
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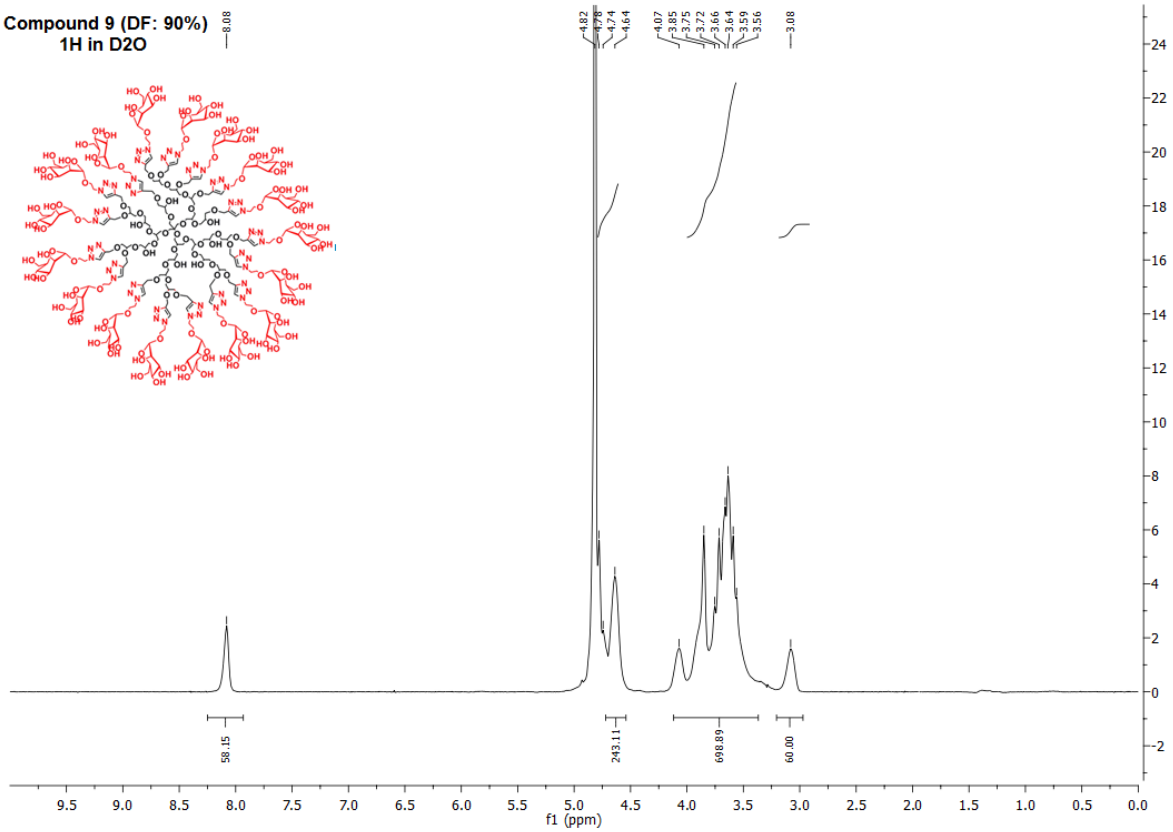
63.02
62.07
61.84
60.26
49.60
29.80
f1 (ppm)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4.78
4.74
4.64
4.07
3.85
3.75
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3.64
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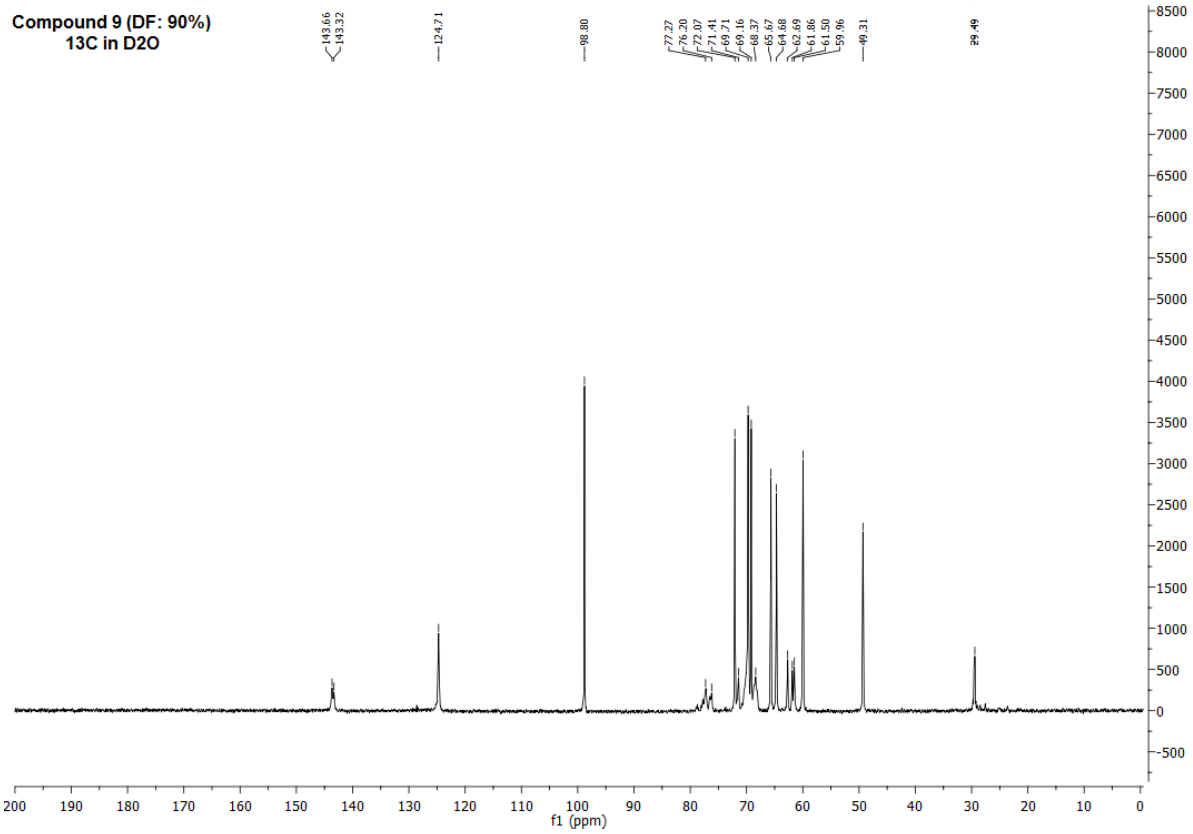
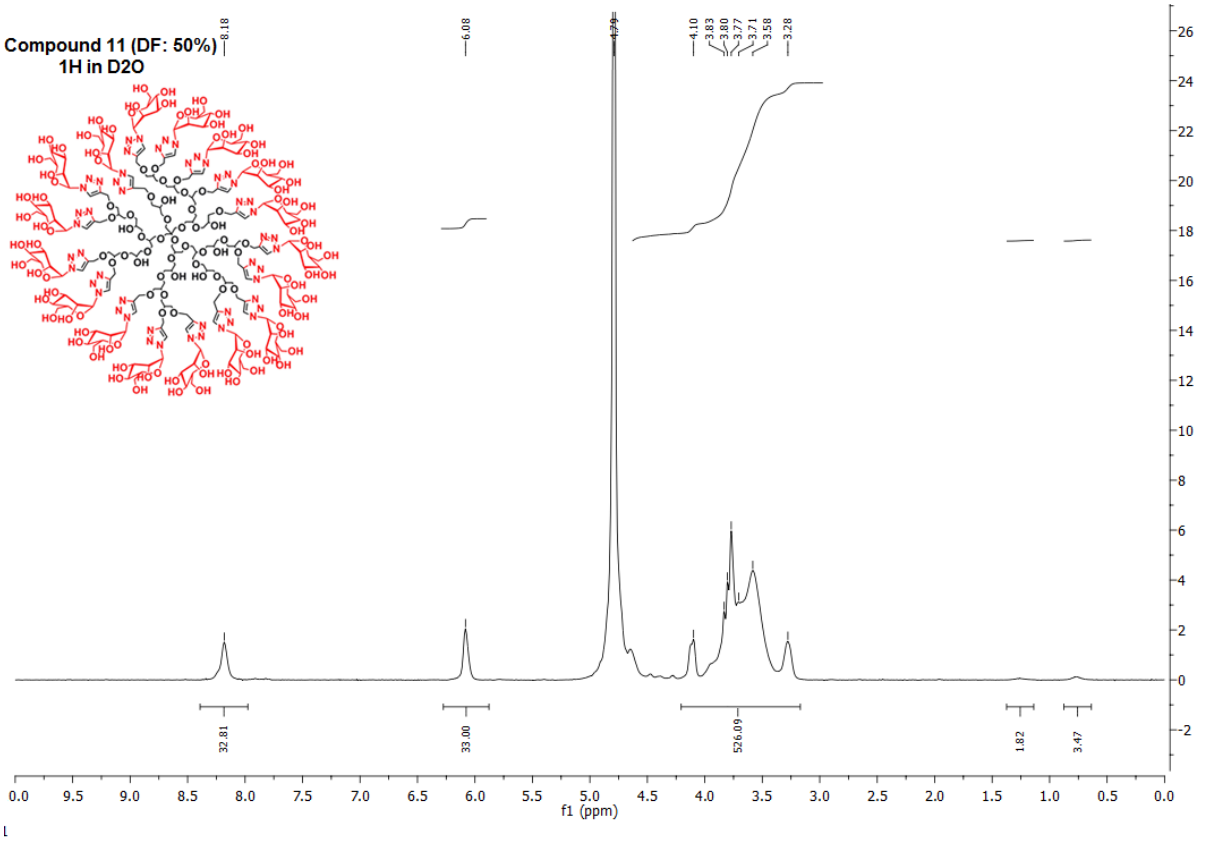
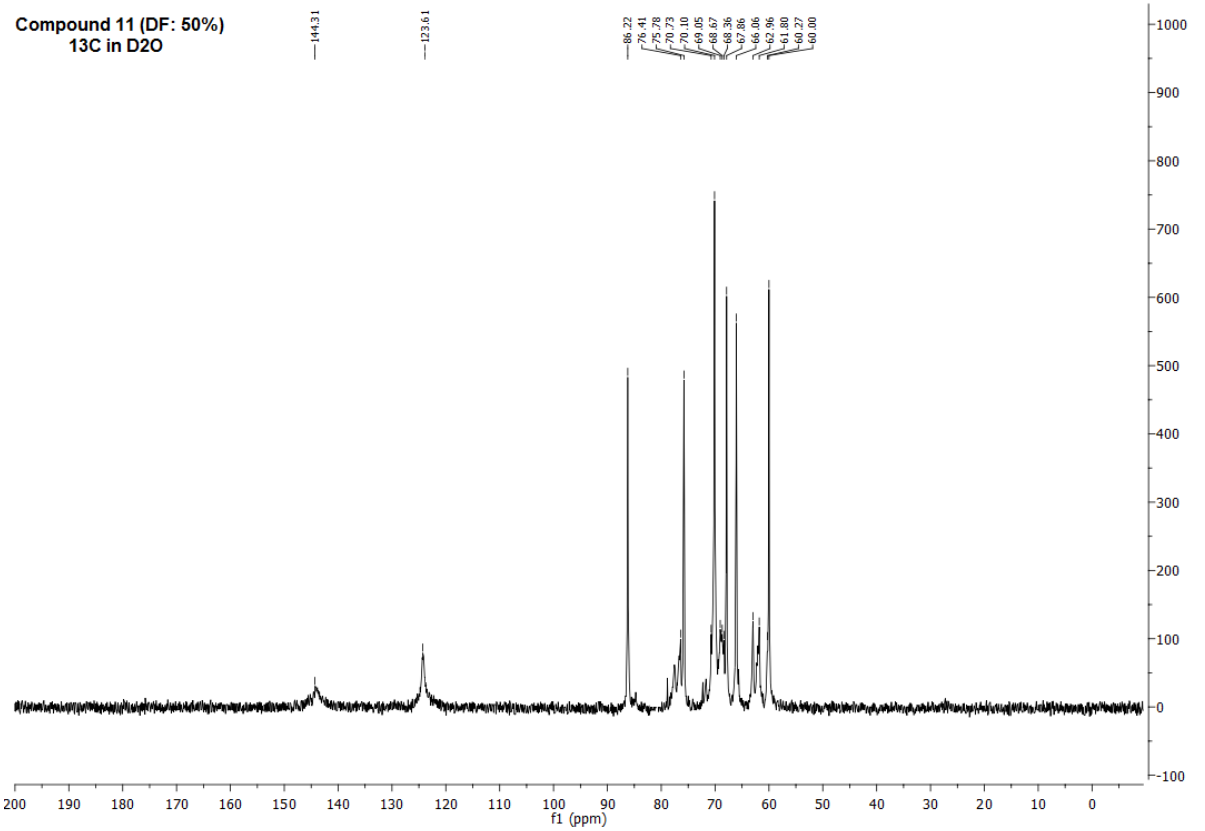
3.56
3.08
58.15
243.11
698.89
60.00
f1 (ppm)


Compound 9 (DF: 90%)
13C in D2O
143.66
143.32
124.71
98.80
77.27
76.20
72.07
71.41
69.71
69.16
68.37
65.67
64.68
62.69
61.86
61.50
59.96
49.31
29.49
f1 (ppm)


Compound 11 (DF: 50%)
1H in D2O
8.18
6.08
4.79
4.10
3.83
3.80
3.77
3.71
3.58
3.28
32.81
33.00
526.09
1.82
3.47
f1 (ppm)


Compound 11 (DF: 50%)
13C in D2O
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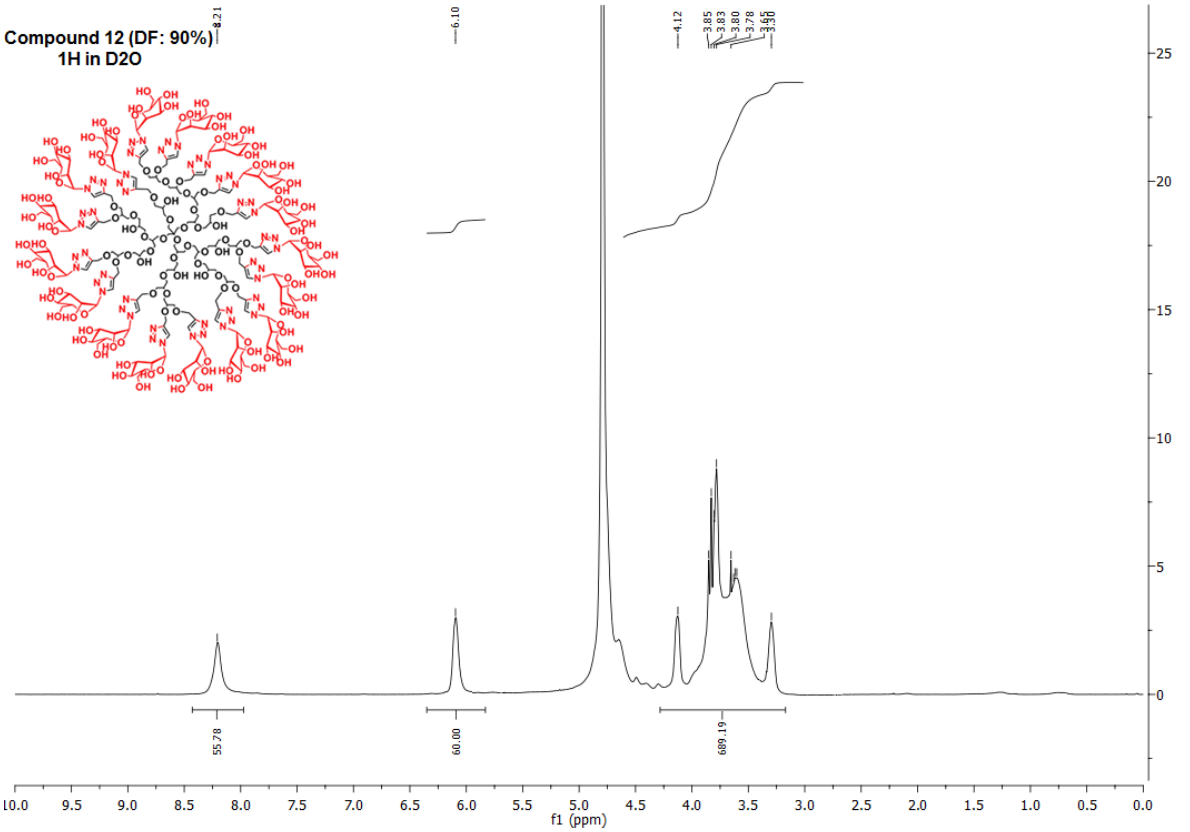
123.61
86.22
76.41
75.78
70.73
70.10
69.05
68.67
68.36
67.86
66.06
62.96
61.80
60.27
60.00
f1 (ppm)


Compound 12 (DF: 90%)
1H in D2O
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6.10
4.12
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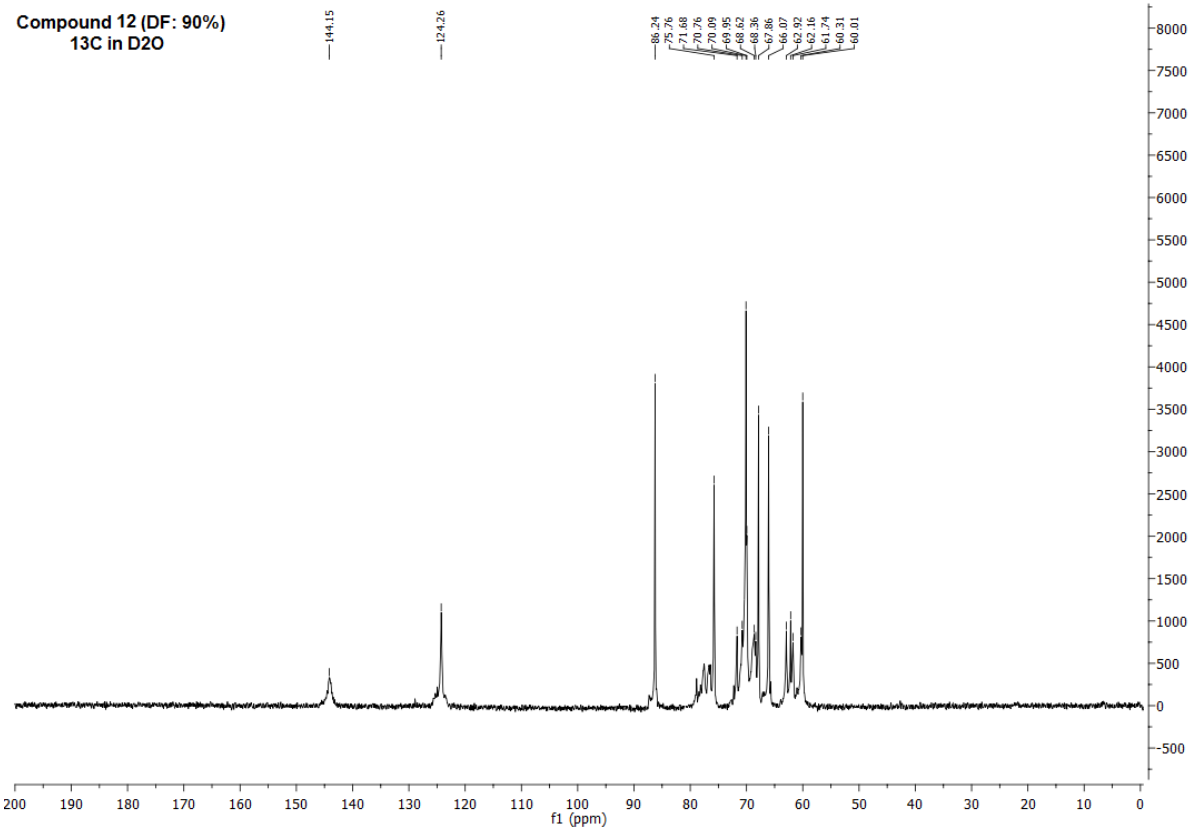
3.83
3.80
3.78
3.65
3.50
55.78
60.00
689.19
f1 (ppm)


Compound 12 (DF: 90%)
13C in D2O
144.15
124.26
86.24
75.76
71.68
70.76
70.09
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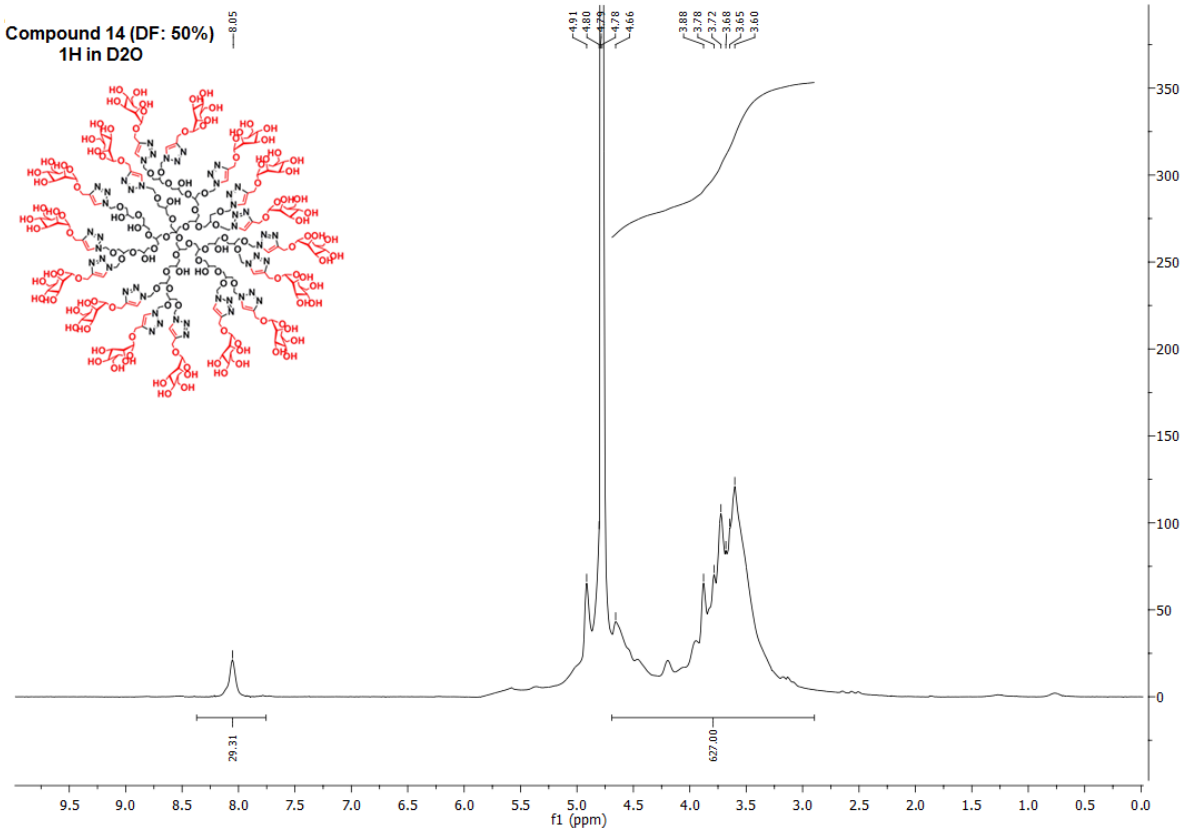
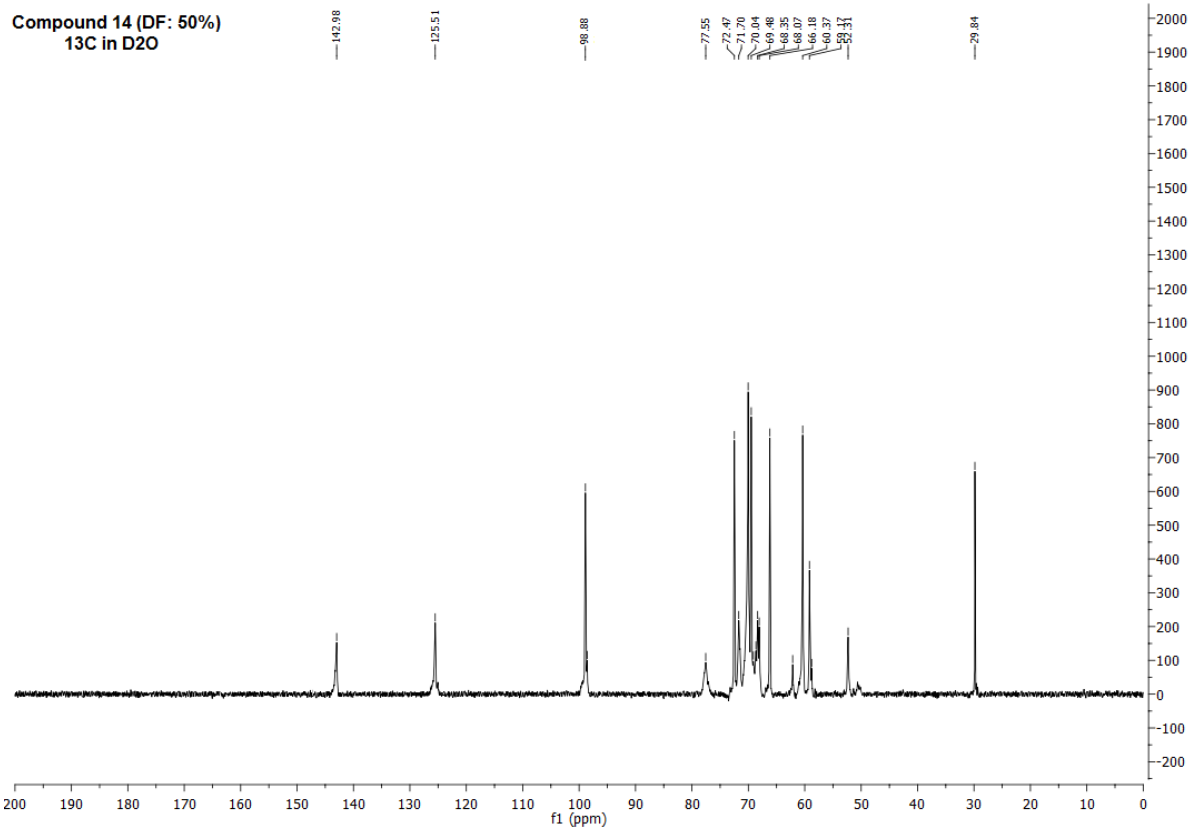
61.74
60.31
60.01
f1 (ppm)


Compound 14 (DF: 50%)
1H in D2O
8.05
4.91
4.80
4.79
4.78
4.66
3.88
3.78
3.72
3.68
3.65
3.60
29.31
627.00
f1 (ppm)


Compound 14 (DF: 50%)
13C in D2O
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125.51
98.88
77.55
72.47
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69.48
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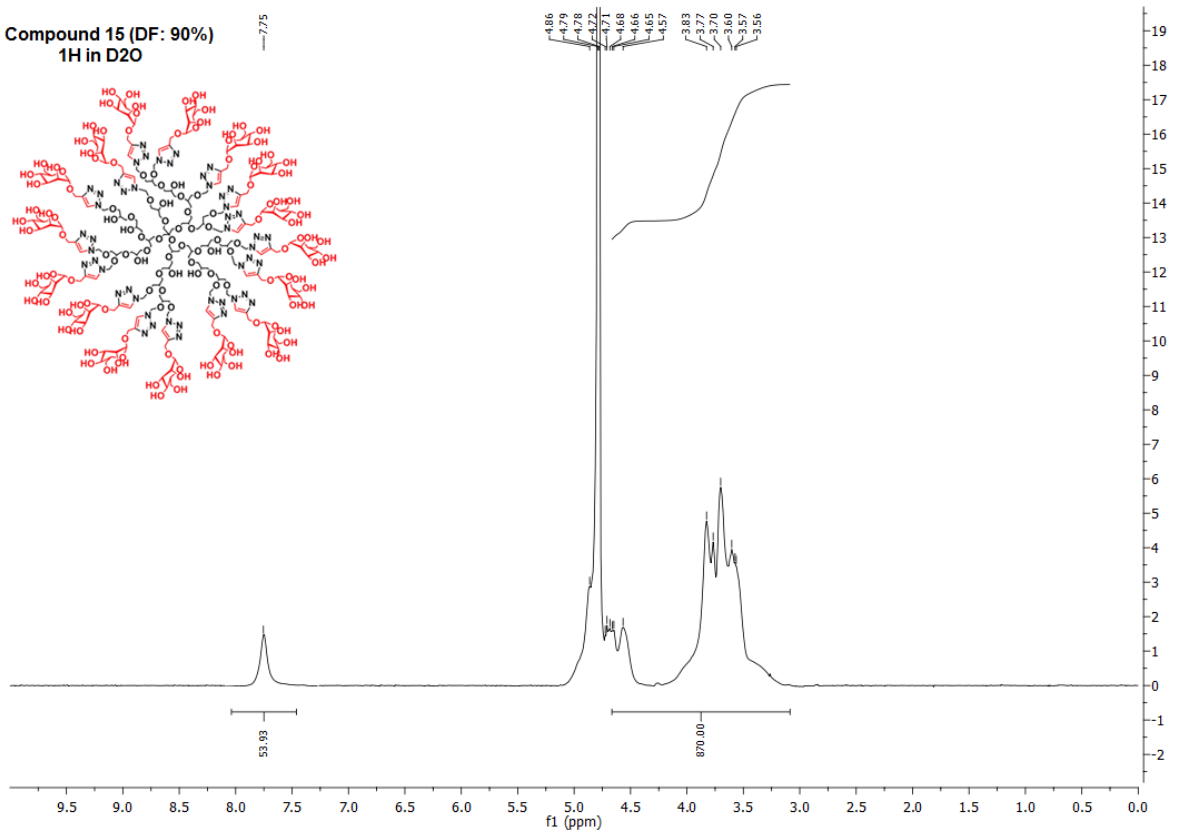
68.07
66.18
60.37
29.84
f1 (ppm)


Compound 15 (DF: 90%)
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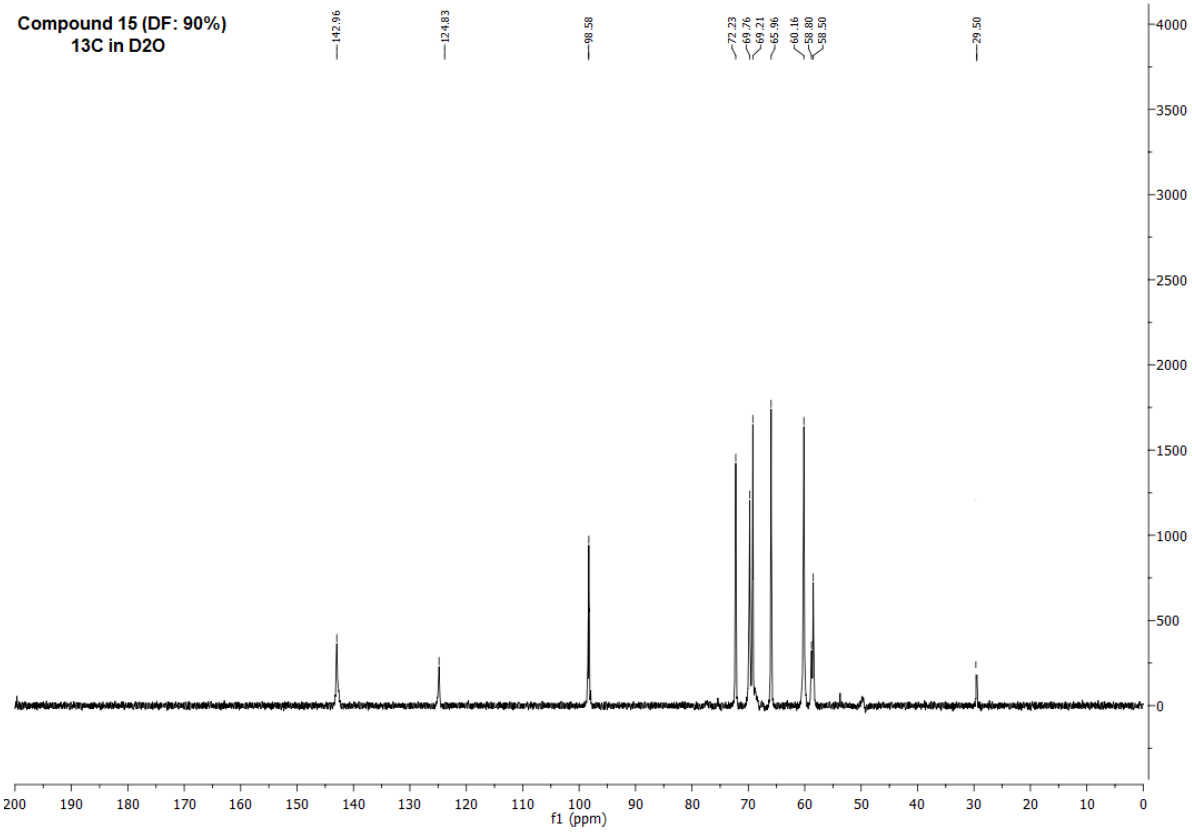
7.75
4.86
4.79
4.78
4.72
4.71
4.68
4.66
4.65
4.57
3.83
3.77
3.70
3.60
3.57
3.56
53.93
870.00
f1 (ppm)


Compound 15 (DF: 90%)
13C in D2O
142.96
124.83
98.58
72.23
69.76
69.21
65.96
60.16
58.80
58.50
29.50
f1 (ppm)