Novel Glycodendrimers Self-Assemble to Nanoparticles which Function as Polyvalent Ligands In Vitro and In Vivo**

Gebhard Thoma,* Andreas G. Katopodis, Nicolas Voelcker, Rudolf O. Duthaler, and Markus B. Streiff

The recognition of oligosaccharides by proteins represents the basis of many biologically important events.[1] Individual protein–carbohydrate interactions are generally weak ($K_D = 10^{-3}$–$10^{-4}\,M^{-1}$).[2] To overcome this, such processes often involve polyvalent binding, which is characterized by the simultaneous contact of multiple ligands (oligosaccharides) on one biological entity to multiple receptors (proteins) on another.[3] Polyvalent carbohydrate–protein interactions occur frequently in recognition events on cellular membranes. Collectively, they can be much stronger than corresponding monovalent interactions rendering it difficult to control them with individual small molecules.[4] Therefore, complex macromolecules have been used as polyvalent antagonists, however, both characterization and preparation of these nonuniform entities is demanding.[4] Here we present an alternative concept for the polyvalent presentation of ligands based on the supramolecular chemistry[5] of small molecules that fulfil single-molecule entity criteria (Figure 1). Novel dendrons capped with carbohydrate ligands (glycodendrimers[6]) were found to self-assemble to noncovalent nanoparticles which function as polyvalent ligands. We demonstrate that these particles—not the individual molecules—efficiently inhibit polyvalent interactions, such as IgM binding (IgM = immunoglobulin), to the αGal-epitope[7] (α-D-Gal-(1→3)-β-D-Gal-(1→4)-D-GlcNAc), both in vitro and in vivo. As self-assembly is dynamic, optimization of size and shape of the polyvalent ligand could occur utilizing the receptor as a template.

Dendrimer cores were prepared by a convergent "outside-in" approach[8] based on a single building block **1a** which was obtained from methyl 3,5-diaminobenzoate and 4-(*tert*-butoxycarbonylaminomethyl)benzoic acid (Scheme 1a). Selective deprotection furnished **1b** and **1c** (first-generation dendrimer core, two end-groups). A one-pot procedure comprising coupling of **1c** (1 equiv) and **1b** (0.5 equiv) followed by methyl ester cleavage gave **2c** (second-generation dendrimer core, four end groups).[9] The third-generation dendrimer **3c** (eight end groups) was obtained from **2c** (1 equiv) and **1b** (0.5 equiv).[9] Applying the same procedure repetitively gave

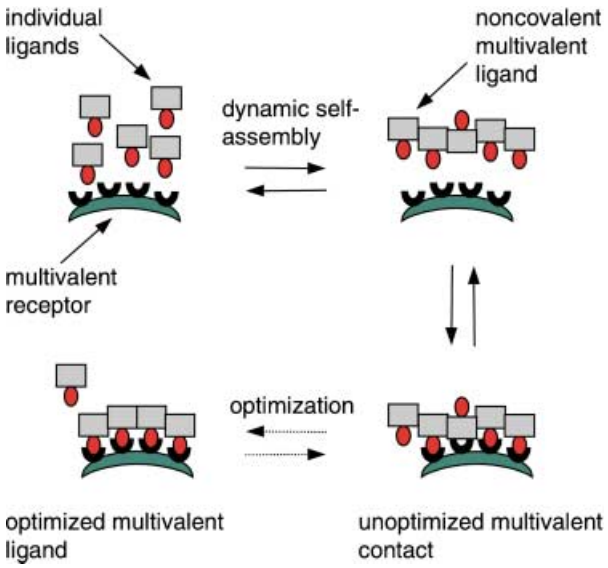


Figure 1. Individual molecules, comprised of both a ligand and a self-assembling moiety, form noncovalent nanoparticles which function as multivalent ligands. If self-assembly is a dynamic process natural polyvalent receptors could serve as templates optimizing size and shape of their own polyvalent inhibitors.

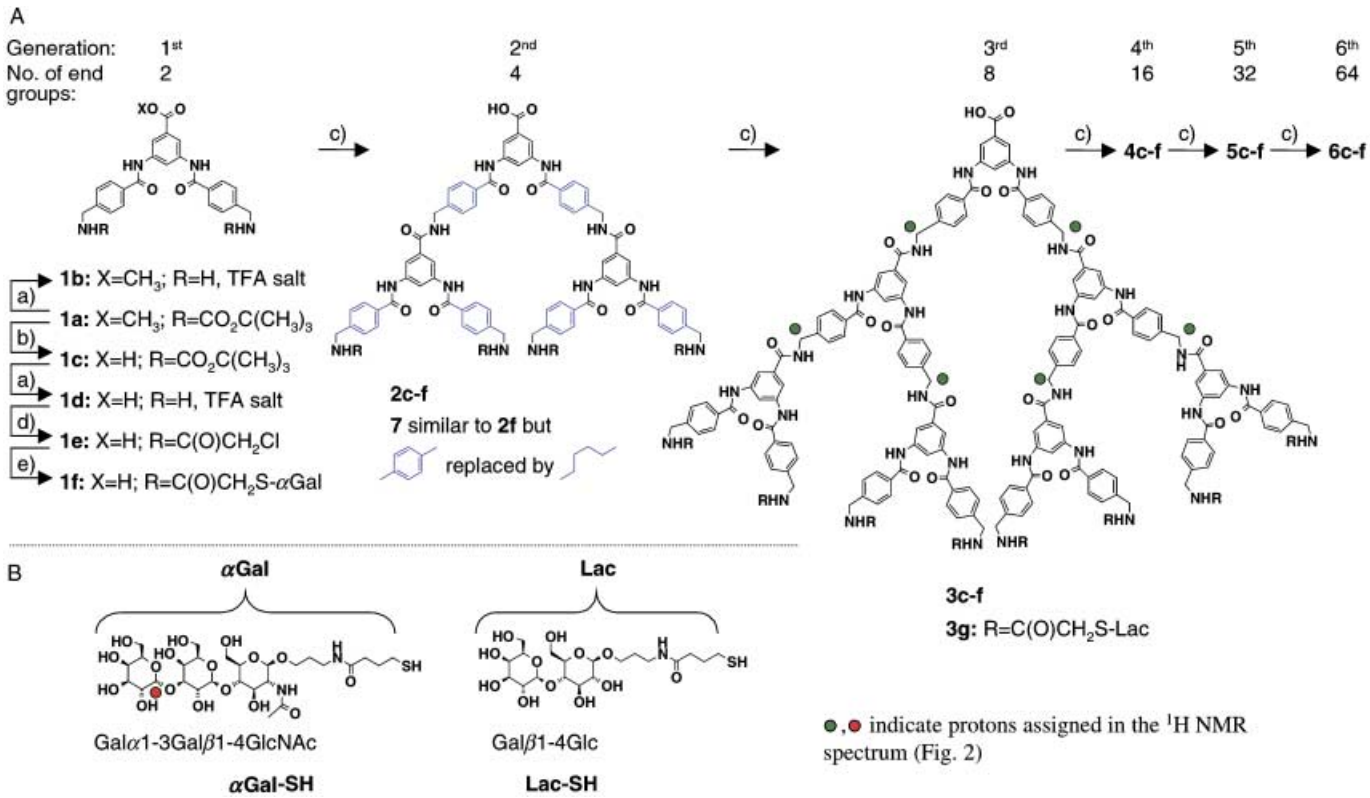
dendrimers with up to 64 end groups (**4c**, fourth generation, 16 end groups; **5c**, fifth generation, 32 end groups; **6c**, sixth generation, 64 end groups). Dendrimers **1c**–**6c** were deprotected (→**1d**–**6d**) and transformed into their chloroacetamide derivatives (**1e**–**6e**) to allow subsequent introduction of thiolated oligosaccharides such as αGal-SH[10] and Lac-SH (Figure 2b) furnishing water-soluble glycodendrimers **1f**–**6f** and **3g** which were purified by ultrafiltration. Compound **7**, which is similar to **2f** but contains butylene chains instead of the disubstituted aromatic rings, was also prepared (Scheme 1a). The integrity of all compounds was established by ^{1}H NMR spectroscopy. Accordingly, the first- to third-generation dendrimers exist as single molecules (purity > 95%). The fourth- to sixth-generation dendrimers possibly contain minute quantities of smaller fragments. The 500 MHz ^{1}H NMR spectra of compound **3f** in [D$_6$]DMSO demonstrates the remarkable purity of these compounds (Figure 2).

The first indication that our glycodendrimers were aggregating in water came from ^{1}H NMR spectroscopy of **2f** in D_2O. At ambient temperature, we observed very broad signals which sharpened at elevated temperatures. The aggregation was quantified using multiangle light scattering (MALS; Table 1). The first-generation dendrimer **1f** forms small aggregates (50 kDa) whereas **2f** forms large particles of 7000 kDa (more than 1500 individual molecules per particle). Interestingly, the particle weight obtained for **3f**–**6f** drops (2200 to 200 kDa) with increasing mass of the individual molecule. The root-mean-square radii of the particles formed by **2f**–**6f** showed the same trend (for **2f**, **3f**, and **4f** 49, 34, and 12 nm, respectively; for **5f** and **6f** the radii were below the detection limit of 10 nm). Core-modified second-generation glycodendrimer **7** (4 × αGal), which is of comparable size and lipophilicity as **2f** but contains butylene chains instead of the disubstituted aromatic rings, does not form aggregates. The third-generation compounds **3g** (8 × Lac; 1900 kDa) and **3f**

[*] Dr. G. Thoma, Dr. A. G. Katopodis, Dr. R. O. Duthaler, Dr. M. B. Streiff
Novartis Pharma AG
P.O. Box, 4002 Basel (Switzerland)
Fax: (+41) 61 324 6735
E-mail: gebhard.thoma@pharma.novartis.com
Dr. N. Voelcker+
The Scripps Research Institute
Beckman Centre for Chemical Sciences
10550, N. Torrey Pines Rd., La Jolla, CA 92034 (USA)

[**] G.T. thanks Prof. M. Reza Ghadiri for the opportunity of a sabbatical stay in his laboratory at the Scripps Research Institute. We also would like to thank Mr. Klaus Menninger for his assistance in the monkey experiment.

  0044-8249/02/4117-3195 $ 20.00+.50/0



Scheme 1. A) Reaction conditions: a) TFA, Et_3SiH, H_2O (95:2.5:2.5), 3 h, RT (>80%); b) 1N NaOH/dioxane (1:1), 16 h, RT (82%); c) 1) **1a** (0.5 equiv), HBTU (1.0 equiv), DMF/iPr_2NEt (4:1), 6–16 h, RT; 2) LiOH, H_2O (>75%); d) chloroacetic acid anhydride (3.0 equiv), DMF/2,6-lutidine (3:1), 3 h, RT (50–95%); e) αGal-SH or Lac-SH (1.5 equiv per end group), DMF, DBU, 1 h, RT; B) structures of αGal-SH and Lac-SH. TFA = trifluoroacetic acid, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, DMF = *N*,*N*-dimethylformamide

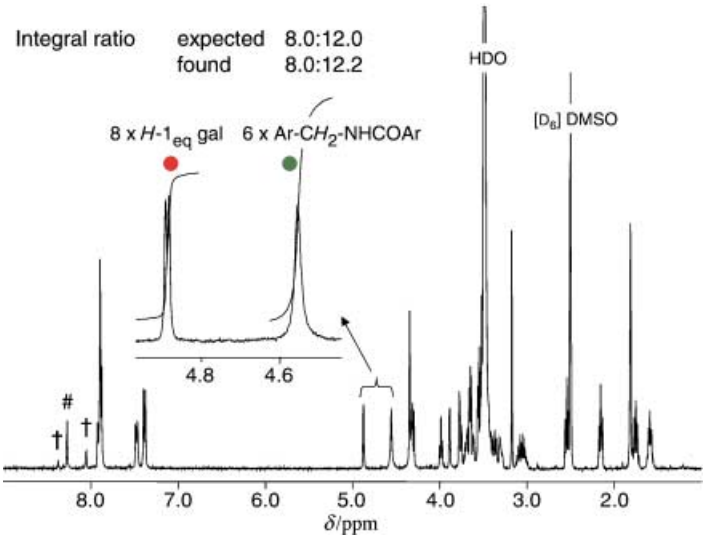


Figure 2. 500 MHz ^1H NMR spectrum of **3f** (third generation, eight end groups) recorded in $[D_6]DMSO/D_2O$ (5:1) at 75°C. The spectrum demonstrates the remarkable purity (>95%) of this compound. The indicated signals represent 8×H-1 of the terminal α-linked galactose residue (see Scheme 1b, red) and 6×Ar-CH_2-NHCO-Ar of the dendrimer core (see Scheme 1a, green). Their relative intensities (8.0:12.2; expected 8.0:12.0) proves the quantitative functionalization with αGal. The small signals (indicated by †) at $\delta = 8.05$ ppm (intensity 1H) and $\delta = 8.38$ ppm (intensity 2H) can be assigned to the protons of the terminal trisubstituted ring of the dendrimer core structure, bearing the carboxylic acid functionality. The signal at $\delta = 8.26$ ppm (intensity 6H, indicated by #) reflects the protons between the N substituents of the six internal trisubstituted rings.

Table 1. Properties of Dendrimers.

Compound (end groups)	M_r[a] (individual molecule) [kDa]	M_r[b] (aggregate) [kDa]	IgM[c] IC_{50} [μM]	Hemolysis[c] IC_{50} [μM]
1f (2)	1.908	50 ± 5	>10	>100
2f (4)	4.198	7100 ± 250	0.025	0.035
3f (8)	8.779	2200 ± 70	0.010	0.010
4f (16)	17.941	1200 ± 50	0.019	0.180
5f (32)	36.264	270 ± 10	>1	0.550
6f (64)	72.911	200 ± 10	>1	2.240
3g (8)	7.154	1900 ± 100	>100	n.d.
7 (4)	4.078	7 ± 1	>100	>100

[a] Satisfactory MALDI mass spectra were obtained for all compounds with the exception of **5f** and **6f**. Interestingly, intense fragmentation was observed for all compounds, even for the first-generation glycodendrimer **1f**. [b] All determinations of the weight-average molar mass (M_r) were performed by Wyatt Technology Deutschland GmbH by multi-angle light scattering (MALS) using a DAWN EOS and a Wyatt Optilab 903 refractometer; at 20°C; concentration 0.3 mg mL^{-1} (0.06 mg mL^{-1} for **2f** as the signal intensity was too high at 0.3 mg mL^{-1} because of high-molecular-weight particles); refractive index increment (d*n*/dc) values were between 0.18 and 0.22 mL g^{-1}. [c] Average of at least two experiments (variations within a factor of 2.5). For assay conditions see bioassays. Concentrations refer to equivalent concentration of trisaccharide but not to the concentration of oligovalent glycodendrimer.

(8×αGal; 2200 kDa), with identical backbones but different carbohydrate end groups, form aggregates of very similar size. Thus, the particle weight strongly depends on the dendrimer core structure but less on the size of the hydrophilic end groups. Intermolecular hydrogen bonds seem not to be significantly involved in self-assembly since particle weights

  0044-8249/02/4117-3196 $ 20.00+.50/0 *Angew. Chem. Int. Ed.* **2002**, *41*, No. 17

are not reduced by the H-bond-disrupting reagent guanidinium hydrochloride (1.5 M), as determined by light scattering. Most probably, the highly aromatic core induces aggregation as a result of π stacking and/or rigidity. The core of **1 f** seems to be too small for efficient core–core interactions whereas the second-generation core of **2 f** is optimal for self-assembly. The decreasing particle weight obtained for higher generation glycodendrimers (**3 f**–**6 f**) could be explained by the increasing number of large end groups per individual molecule inducing a more globular shape. As a consequence, the core is more efficiently shielded by carbohydrates, which renders intermolecular core–core contact more difficult. The particle weight does not substantially depend on the concentration, as shown for dendrimer **2 f** (7000 kDa at 0.06 mg mL^{-1}, 6000 kDa at 0.03 mg mL^{-1}, 3700 kDa at 0.01 mg mL^{-1}), but is significantly affected by temperature (Table 2); it drops ten fold with an increase in temperature to 70 °C, but increases as the temperature is lowered. Comparable particle weights were determined at identical temperatures for both the heating and the cooling period which indicates there is a dynamic self-assembly process which rapidly reaches thermodynamic equilibrium. Both the shape and size of the formed nanoparticles was investigated by atomic force microscopy (AFM). Aqueous solutions of **2 f**–**6 f** were applied to mica surfaces and the solvent evaporated. The observed particles have a disklike morphology (Figure 3), and their average diameters decrease with increasing mass of the individual molecules (61 nm, **2 f**; 13 nm, **6 f**), thus showing the same trend as the molecular weights in solution. The size distribution for the individual generations was found to be remarkably homogenous (within a factor of two).

Table 2. Effect of temperature on the weight of nanoparticles formed by **2 f**.

T	Heating period[a] M_r [kDa]	Cooling period[b] M_r [kDa]
30 °C	5000 ± 500	4600 ± 500
50 °C	2200 ± 300	2600 ± 500
70 °C	550 ± 100	350 ± 50

[a] A cold solution of **2 f** (0.03 mg mL^{-1}) was slowly heated to the indicated temperature and after 15 min equilibration time was injected into a thermostat-controlled cell. [b] A hot solution of **2 f** (0.03 mg mL^{-1}) was slowly cooled down to the indicated temperature and after 15 min equilibration time was injected into a thermostat-controlled cell.

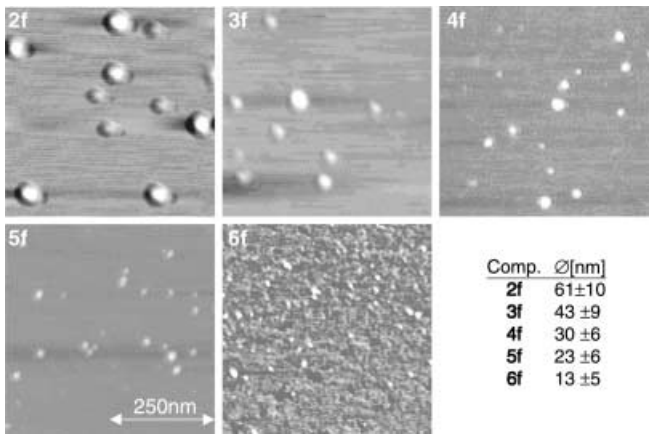


Figure 3. AFM images of particles formed by compounds **2 f**–**6 f**. The size of the particles decreases with increasing molecular weight of the individual glycodendrimer. Aqueous solutions (10 μL; 2 μg mL^{-1} for **2 f**–**5 f**, 0.2 μg mL^{-1} for **6 f**) were spotted on freshly cleaved mica. The solvent was allowed to evaporate for at least 120 min. A Nanoscope III microscope (Digital Instruments) was used to generate the images. The microscope was operated in tapping mode in air with Olympus etched silicon probes (cantilever length 120 μm, tip height 10 μm). The particles exhibit disklike morphologies. Their flatness (1–5 nm) might be caused by interactions with the mica surface. The diameters (∅) were determined by measuring over 30 randomly selected particles.

To exemplify the use of our glycodendrimers as noncovalent multivalent ligands we selected the inhibition of decavalent IgMs directed against the αGal xenoantigen.[7] This epitope is expressed on the cells of mammals, with the exception of humans and Old World monkeys. Anti-αGal antibodies are the most abundant natural human antibodies and cause the hyperacute rejection of pig organs transplanted into primates. They include IgG, IgM, and IgA classes with the IgM class being dominant in the immediate, complement-dependent, hyperacute destruction of the xenograft. Currently there are no pharmacological agents that affect the production of this class of antibodies and research efforts have focused on blocking or removing anti-αGal antibodies prior to pig-to-primate xenotransplantation. Recently, it has been demonstrated that polymers providing multiple copies of the αGal epitope can inhibit IgM binding in vitro.[10,11] To assess our compounds as polyvalent IgM ligands we employed two in vitro assays in which the inhibition of both the anti-αGal IgM binding to the xenoantigen[12a] and the αGal-mediated lysis of pig erythrocytes[13a] were measured (Table 1). In both assays monomeric αGal was inactive at 100 μM, which is in agreement with the finding that individual carbohydrate–protein interactions are often weak.[2] Divalent first-generation dendrimer **1 f** which forms small aggregates (50 kDa) also showed no effect in the binding assay. The second- and third-generation dendrimers **2 f** and **3 f**, which form large nanoparticles, were highly potent in both assays (0.025, 0.035 μM and 0.010, 0.010 μM, respectively).[14] Compound **4 f** also showed high potency in the binding assay (0.019 μM) but was significantly less potent in the hemolysis assay (0.18 μM). Potency dropped even more for the larger dendrimers **5 f** and **6 f** which do not aggregate. Thus, especially in the more relevant hemolysis assay, potency clearly correlates with the size of the aggregates but not with the size of the individual molecules. The inhibition data suggest that the optimal particle weight and size for IgM inhibition are obtained with **3 f**. The particles formed by **2 f** might be larger than the optimum, but still displayed high potency. The aggregates formed by **1 f**, **4 f**, **5 f**, and **6 f** are apparently too small to accomplish polyvalent amplification of IgM inhibition. Further evidence for the involvement of polyvalent aggregates (but not oligovalent dendrimers) in antibody binding stems from the observation that backbone-modified tetravalent αGal dendrimer **7** (size and lipophilicity comparable to **2 f**), which does not aggregate, was found to be inactive whereas **2 f** was highly potent.[15] Nonspecific interactions of the dendrimer backbone can be excluded because dendrimer **3 g** (identical backbone and similar aggregation

  0044-8249/02/4117-3197 $ 20.00+.50/0

properties as **3f** but Lac instead of αGal) did not inhibit binding. The most potent compound **3f** was selected for in vivo profiling in cynomolgus monkeys (1 mg kg^{-1}, intravenous, $n=3$; Figure 4). Within five minutes after injection the anti-αGal IgMs, as detected by enzyme-linked immuno

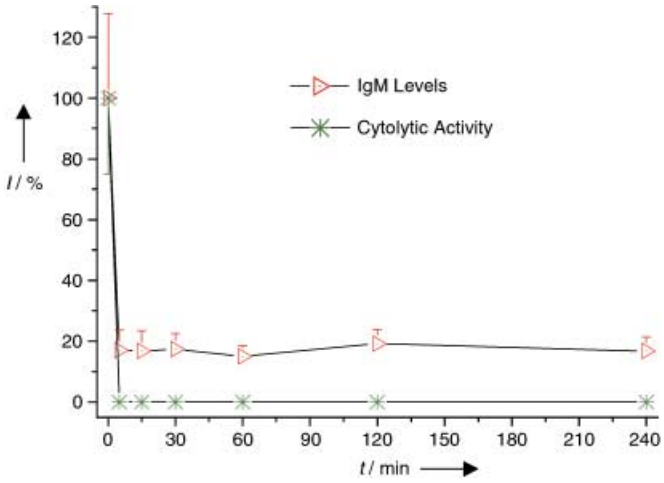


Figure 4. Effects of **3f** on anti-αGal IgM levels and cytolytic activity of the sera of cynomolgus monkeys ($n=3$; 1 mg kg^{-1}; intravenous; plasma concentration approximately 10 μM assuming all of **3f** is present). Prior to injection ($t=0$) the IgM 100% level (I) was 2.3 times higher than the IgM level of pooled human sera.

sorbent assay (ELISA), were reduced to 20% of the initial value and remained at low levels for more than 4 h.[12b] Most importantly, anti-αGal antibody-mediated haemolytic activity was completely eliminated.[13b]

In conclusion, we have described novel, self-assembling glycodendrimers which form noncovalent nanoparticles in water. The particle sizes are remarkably homogenous and can be varied over a broad range by the appropriate choice of the dendrimer generation. The particles can be deposited on surfaces by evaporation of the solvent. In solution, they function as noncovalent polyvalent inhibitors both in vitro and in vivo. The self-assembly of the glycodendrimers is a dynamic equilibrium process. Thus, it is conceivable that noncovalent polyvalent ligands are optimized with respect to size and shape in the presence of natural polyvalent receptors. The control of a broad variety of physiologically relevant polyvalent interactions should be possible.

Received: April 16, 2002 [Z19101]

[1] a) A. Varki, *Glycobiology* **1993**, *3*, 97; b) R. A. Dwek, *Chem. Rev.* **1996**, *96*, 622.

[2] Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321.

[3] a) L. L. Kiessling, N. L. Pohl, *Chem. Biol.* **1996**, *3*, 71; b) R. Roy, *Trends Glycosci. Glycotechnol.* **1996**, *8*, 79.

[4] M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754.

[5] J. M. Lehn, *Supramolecular Chemistry*, VCH, Weinheim, **1995**.

[6] Glycodendrimers have been described earlier. See, for example, a) N. Rockendorf, T. K. Lindhorst, *Top. Curr. Chem.* **2001**, *217*, 201; b) R. Roy, M.-G. Baek, K. Rittenhouse-Olson, *J. Am. Chem. Soc.* **2001**, *123*, 1809; c) B. Colonna, V. D. Harding, S. A. Nepogodiev, F. M. Raymo, N. Spencer, J. F. Stoddart, *Chem. Eur. J.* **1998**, *4*, 1244.

[7] a) *Subcellular Biochemistry, Vol. 32* (Eds.: U. Galili, J. L. Avila), Kluwer Academic/Plenum, New York, **1999**; b) M. S. Sandrin, I. F. C. McKenzie, *Immunol. Rev.* **1994**, *141*, 169.

[8] a) J. M. J. Fréchet, *Science* **1994**, *263*, 1710; b) G. R. Newkome, C. N. Moorefield, F. Vögtle, *Dendritic Molecules: Concepts, Syntheses, Perspectives* VCH, Weinheim, **1996**.

[9] Ethyl diisopropylamine (3 mL) was added to a mixture of **1b** (0.50 equiv), **1c–5c** (1.00 g; 1.00 equiv), and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) (1.00 equiv) in dry DMF (10 mL). The solution was stirred for 16 h at room temperature. DMF (5 mL), water (3 mL, dropwise), and LiOH monohydrate (approximately 50 equiv) were added and the mixture was stirred for an additional 16 h at room temperature. The solution was added dropwise to a mixture of acetone and 0.2 N HCl (250 mL, 1:3). The precipitate formed was filtered off and washed with water, acetone, and dichloromethane. Products **2c–6c** (all yields greater than 80%) were isolated as beige solids.

[10] R. Duthaler, A. Katopodis, W. Kinzy, R. Ohrlein, G. Thoma, WO 9847915 **1998**; [*Chem. Abstr.* **1998**, *129*, 331058].

[11] J.-Q. Wang, X. Chen, W. Zhang, S. Zacharek, Y. Chen, P. G. Wang, *J. Am. Chem. Soc.* **1999**, *121*, 8174.

[12] ELISAs**:** a) Human serum: Wells on plate A were coated with 0.5 μg of αGal-HSA (HSA = human serum albumin) conjugate in $NaHCO_3$ (100 μL, 100 mM; pH 9.6) for 2 h at 37 °C. Blocking with phosphate buffer saline (PBS, 250 μL) containing 0.5% Tween 20 for 2 h at ambient temperature was followed by removal of the liquid. On plate B pooled human serum was diluted (1:20) with PBS containing 10% Seablock and 0.2% Tween 20 and incubated for 30 min with serial dilutions of dendrimers or other inhibitors. Samples (100 mL) from each well on plate B were transferred to plate A, incubated for 1 h at ambient temperature, and washed three times with PBS containing 0.15% Tween 20. They were then incubated with PBS containing anti-IgM secondary antibody peroxidase conjugate (1/2000 diluted, 100 μL), 10% Blocker-BSA, and 0.2% Tween 20. After washing with PBS containing 0.1% Tween 20 (6 × 300 μL), color development was initiated by adding TMB substrate solution (100 μL, a mixture of 3,3′,5,5′-tetramethylbenzidin, H_2O_2, and stabilizers) and stopped after 5 min with 1 M H_2SO_4 (50 μL). The absorbance at 450 nm (A_{450}) was measured; b) Cynomolgus monkey serum: wells were coated with αGal-polymer[10] (0.5 μg) in PBS (100 μL) overnight at 4 °C. Blocking with PBS containing 10% Seablock (300 μL) for 2 h at ambient temperature was followed by removal of the liquid. The wells were incubated for 30 min with 100 μL of serially diluted serum sample in PBS containing 10% Seablock and 0.2% Tween 20 and, subsequently, washed three times with PBS containing 0.15% Tween 20. Incubation with a secondary antibody and color development were performed as described above.

[13] Hemolysis assays. a) Human serum: pig erythrocytes were obtained from heparinized pig blood, washed three times with CFD buffer (3.1 mM diethyl barbituric acid, 0.9 mM sodium barbitone, 145 mM NaCl, 0.83 mM $MgCl_2$, 0.25 mM $CaCl_2$; pH 7.4), and suspended in CFD at a concentration of 1×10^9 mL^{-1}. Seven serial dilutions of inhibitor were prepared on low-bind U-shaped plates in CFD (50 μL). Human serum was serially diluted in CFD (50 μL, 10 dilutions assayed for each inhibitor concentration). Then, pig erythrocyte solution (50 μL) was added to each well and the plate incubated for 60 min at 37 °C with mild shaking. Plates were centrifuged for 10 min at 2000g to precipitate the unlyzed erythrocytes, the supernatant was transferred to a flat-bottomed plate, and the released hemoglobin (A_{420}) was measured; b) Cynomolgus monkey serum: the assay was carried out as above, but with the addition of baby rabbit complement (50 μL, heterologous complement source at a final dilution of 1:10) in CFD and pig erythrocyte solution (50 μL) to each well.

[14] Polyvalent amplification was found to be less pronounced for the inhibition of divalent IgG binding. Compounds **1f–6f** showed very similar IC_{50} values in the range of 0.45–0.85 μM. Compound **3g** gave no inhibition at 10 μM whereas **7** showed an IC_{50} value of 1.80 μM.

[15] It also has to be considered that the individual molecules **2f** (M_r 4 kDa) and **3f** (9 kDa) are too small to allow for simultaneous contact of several oligosaccharide ligands with several binding sites of an individual IgM molecule (900 kDa).

  0044-8249/02/4117-3198 $ 20.00+.50/0