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Artificial Golgi Apparatus: Globular Protein-like Dendrimer Facilitates Fully Automated Enzymatic Glycan Synthesis

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Abstract: Despite the growing importance of synthetic glycans as tools for biological studies and drug discovery, a lack of common methods for the routine synthesis remains a major obstacle. We have developed a new method for automated glycan synthesis that employs the enzymatic approach and a dendrimer as an ideal support within the chemical process. Recovery tests using a hollow fiber ultrafiltration module have revealed that monodisperse G6 (MW = 58 kDa) and G7 (MW = 116 kDa) poly(amidoamine) dendrimers exhibit a similar profile to BSA (MW = 66 kDa). Characteristics of the globular protein-like G7 dendrimer with high solubility and low viscosity in water greatly enhanced throughput and efficiency in automated synthesis while random polyacrylamide-based supports entail significant loss during the repetitive reaction/separation step. The present protocol allowed for the fully automated enzymatic synthesis of sialyl Lewis X tetrasaccharide derivatives over a period of 4 days in 16% overall yield from a simple *N*-acetyl-D-glucosamine linked to an aminooxy-functionalized G7 dendrimer.

Introduction

It is clear that large amounts of structurally defined homogeneous samples of nucleic acids and peptides have greatly promoted basic research and pharmaceutical/medical applications of these biomolecules. Solid-phase chemical synthesis for peptides proposed by Merrifield[1] was also utilized for oligonucleotide (DNA/RNA) synthesis, and enabling the subsequent use of automated synthesizers based on this methodology. As is the case of glycans, which is the third major class of biopolymers next to nucleic acids and proteins, solid-phase chemical synthesis of oligosaccharides has also been studied[2,3] since it was first reported by Frechet and Schuerch[4] in 1971 and, Seeberger et al. developed the first automated carbohydrate synthesizer based on a solid-phase chemical strategy in 2001.[5] However, solid-phase chemical synthesis of oligosaccharides still involves a longstanding characteristic problem. Although extensive efforts have been made toward the development of practical chemical synthetic protocols since the advent of these powerful glycosylation techniques, stereoselective and regioselective glycosylations definitely require multistep syntheses for selective protections/deprotections of the multiple hydroxyl groups present on individual sugars.[6−8] Taking the ability to access the homogeneous glycans into account, these disadvantages are extremely serious for the sequential chemical synthesis on solid materials because purification of products is impossible until the final cleavage from their solid supports. Furthermore, tedious/time-consuming procedures are also needed for the removal of multiple byproduct attached to solid supports such as α/β stereoisomers, regioisomers, unreacted intermediates and so on. On the contrary, enzyme-assisted synthesis is an attractive alternative to chemical synthesis because there is the advantage of accomplishing regio- and stereoselective glycosylations for versatile glycoconjugate acceptors according to the substrate specificity and/or tolerance inhered to enzymes.[9−11] Although the scope of glycan synthesis by mammalian glycosyltransferases is still restricted due to the limitation of available recombinant glycosyltransferases with satisfactory activity and stability required for synthetic purposes, it is generally believed that enzymes from various microbial sources should provide a

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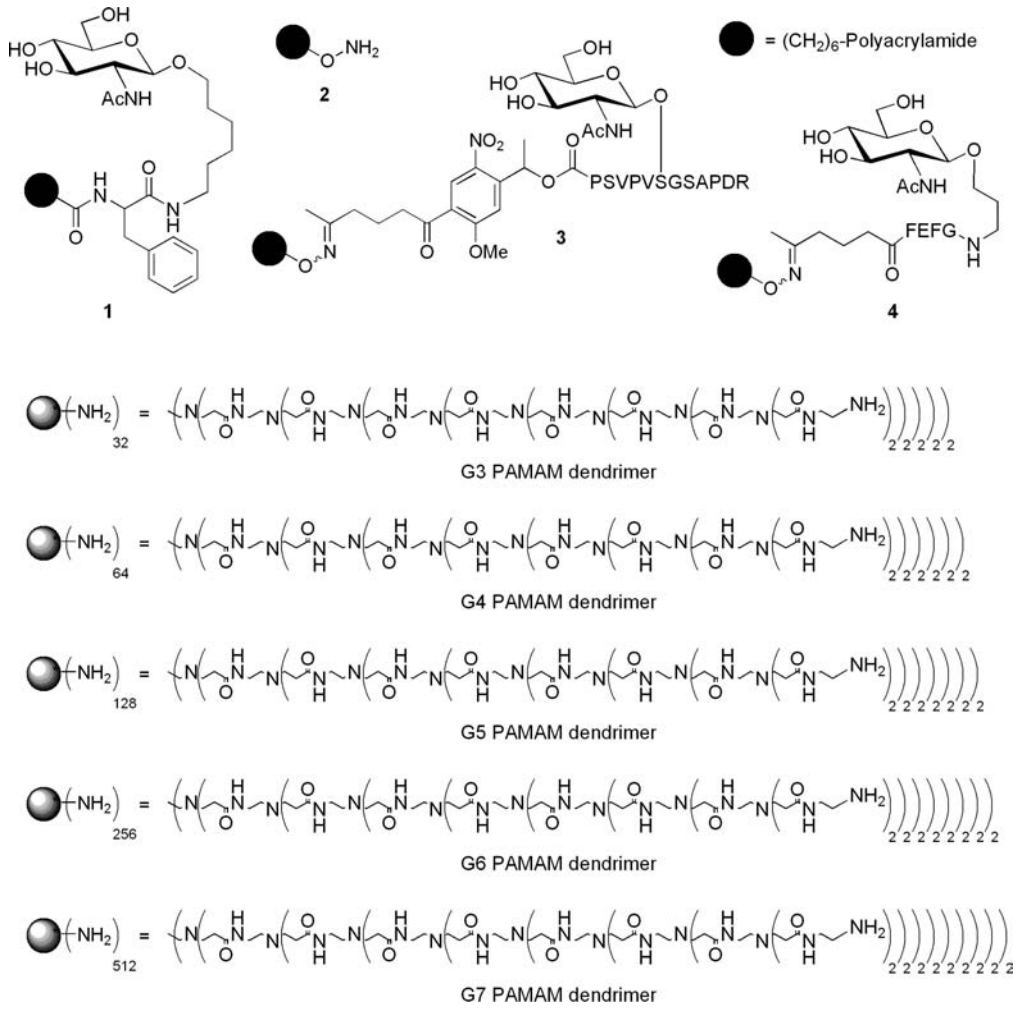

Figure 1. Polymers used for the recovery tests in the size-exclusion separation using a hollow fiber UF module.

promising alternative to mammalian glycosyltransferases for the construction of a wide range of glycan analogues.[12−16]

Toward the automated synthesis of glycoconjugates based on chemical and enzymatic approaches, we have developed a seamless protocol from chemical to enzymatic synthesis by applying water-soluble polymers for supporting various glycosyl acceptor substrates.[17−24] Our recent strategy focusing on the glycopeptide synthesis is as follows: (i) chemical synthesis of designated glycosyl acceptors composed of small sugars such as mono-, di-, or trisaccharide moiety and a heterobifunctional linker that can shuttle between solid-phase and soluble polymer platforms through specific catch and release reactions,[21−24] (ii) chemical ligation of the shuttles with water-soluble polymers resulting in the primer polymers, (iii) sugar elongation reactions by some recombinant glycosyltransferases, and (iv) releasing

Table 1. Summary of the Recovery of Various Water-Soluble Polymers in UF Separation Test

polymer	MW (kDa)	recovery (%)	permeate/loss (%)
BSA	66	85	15
PAA	80	10	90
1	26	52	48
2	82	0	100
3	93	82	18
4	92	0	100
G3 PAMAM dendrimer	7	1	99
G4 PAMAM dendrimer	14	3	97
G5 PAMAM dendrimer	29	12	87
G6 PAMAM dendrimer	58	85	15
G7 PAMAM dendrimer	116	86	14

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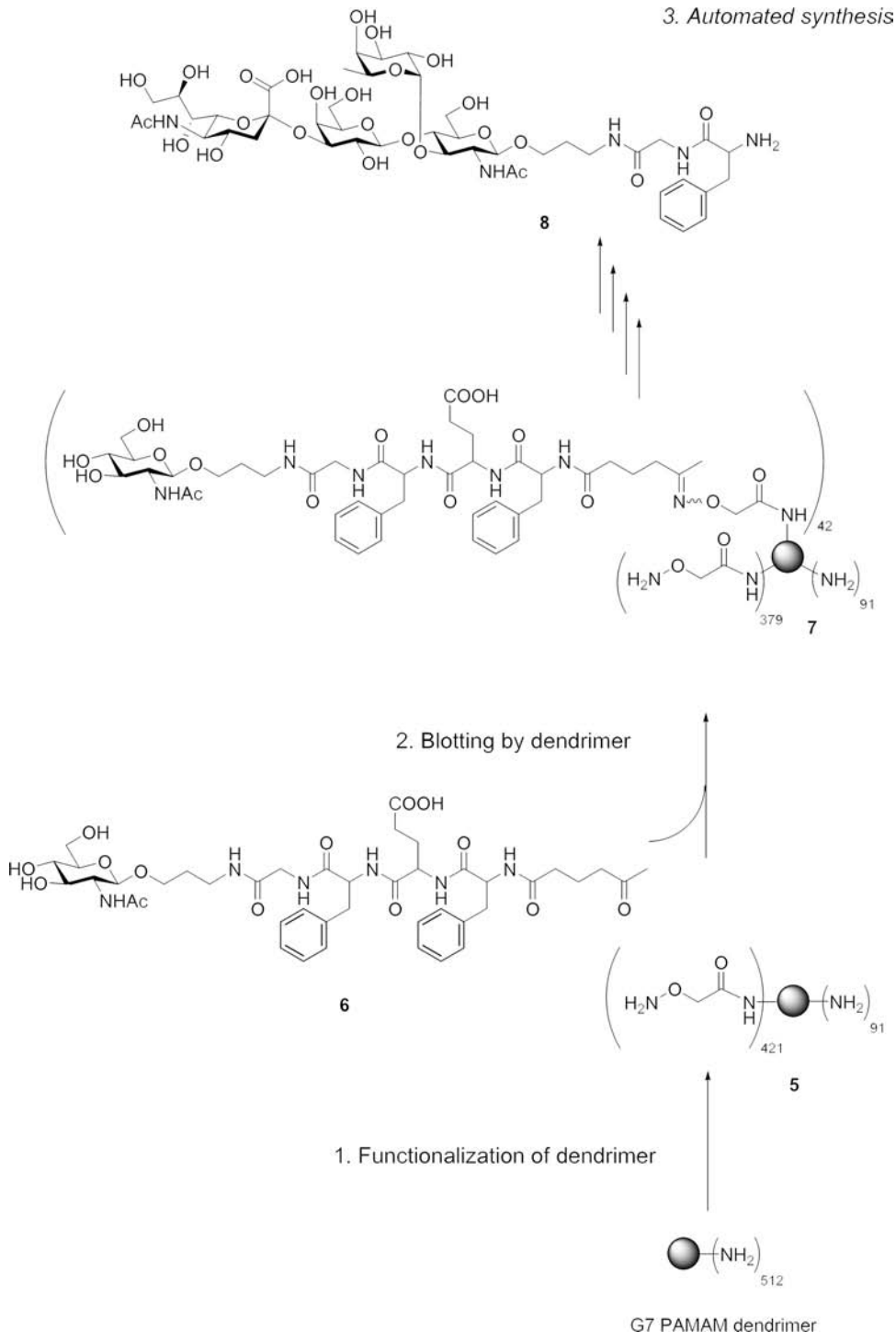

Figure 2. Schematic procedure of automated glycan synthesis supported by aminooxy-functionalized G7 PAMAM dendrimer.

final products from polymer supports by photo-[21] or protease-selective cleavage[22−24] of the linkers and purification. To mechanize steps (iii) and (iv) in a repetitive manner, we have developed a HPLC-based glycan synthesizer, namely, Golgi (Supporting Information, Figure S1). Once a primer polymer is set in the system, designed glycan derivatives or glycopeptides can be synthesized automatically through four designated functions under a computer control: (a) *reaction*, mixing samples with reagents by an autosampler; (b) *separation*, ultrafiltration (UF) of the reaction mixture by using a hollow fiber module to separate polymers bearing products and surplus reagents; (c) *detection*, quantitative monitoring of eluants from diafiltration by UV or fluorescent detectors; and (d) *repetition*, repeating the process for reactions and separations. Among these four basic functions of this system, it was uncovered that the efficiency of the *separation* is a crucial step because the loss during these repetitive purification steps should directly influence the outcome of automated synthesis. In fact, it was suggested that solution property of polyacrylamide (PAA)-based polymer supports strongly affects the overall yields of multistep enzymatic synthesis which is significantly dependent on the structures of proximal moiety linking to glycans (data not shown). This feature of PAA-based polymers has often been a serious hurdle with regard to the achievement of the efficient and practical

automated synthesis of various glycoconjugates. Here we hypothesized that the use of monodisperse and spherical dendrimers[25] having suitable molecular size and surface functional groups might break through the above obstacle during repetitive reaction/separation steps in automated synthesis.

Results and Discussion

Globular Protein-like Properties of Dendrimers. Our interest was focused on the feasibility of a poly(amidoamine) (PAMAM) dendrimer[26] as an alternative candidate as a supporting polymer due to its high monodispersity and spherical molecular shape in addition to the feasibility of attaching surface ligand groups. To assess the effect of molecular size, shape, and dispersity of water-soluble polymers on the efficiency in the separation step using a hollow fiber UF module (Microkros, 10 kDa MWCO), the recovery of PAMAM dendrimers with different generations (G3−G7) were evaluated in comparison with common PAA and PAA-based polymers **1**−**4**[19,21,23] (Figure 1) and bovine serum albumin (BSA) as a control for the monodisperse biopolymer. The results summarized in Table 1 indicate that PAA and polymers **1**−**4** showed a wide range of recovery/retentate from 0 to 82% while BSA (MW = 66 kDa) as a standard retained in 85%. It seems likely that permeability of PAA-based polymers strongly depends on the solubility and viscosity defined by molecular shape and dispersity rather than the molecular weights of the core PAA, since PAA (MW = 80 kDa) retained only 10% while polymer **1** (MW = 26 kDa) showed 52% recovery. Judging from the significant difference of the recovery observed in the polymers bearing β-GlcNAc residues through the similar hydrophobic linkers such as **1** (52%), **3** (82%), and **4** (0%), solution property of this class of polydisperse random polymers appears to be highly sensitive even to the minor changes in chemical structure of the linker moiety. These characteristics of PAA-based polymers appear to significantly affect the yields of enzymatic reactions as well as the recovery in the repetitive separation steps. It should be noted that there is no plausible theory or tendency to predict the feasibility of the PAA-based polymers designed for individual target glycoconjugates in automated glycan synthesis on the Golgi apparatus. Although a few successful automated syntheses were achieved for the construction of cancer-relevant mucin glycopeptide libraries based on the functions of this system,[22,24] limitations due to unfavorable solution properties of PAA-based primers still exist in enzyme-assisted automated syntheses for a wide range of glycoconjugates. On the other hand, PAMAM dendrimers showed excellent profiles in this experiment, in which the G3−G7 dendrimers tested could be divided into two subgroups by a clear threshold between G6 and G5 dendrimers. It was demonstrated that G6 (MW = 58 kDa) and G7 (MW = 116 kDa) retained 85 and 86% while G3 (MW = 7 kDa), G4 (MW = 14 kDa), and G5 (MW = 29 kDa) entirely permeated this UF module. These results indicate that the profiles of G6 and G7 dendrimers are quite similar to those of BSA (MW = 66 kDa), a typical globular protein 2.5 nm in diameter containing a single polypeptide chain 50 nm long that is coiled and folded into a compact bundle. This clearly means that the efficiency in the size-exclusion separation of water-soluble polymers by means of the hollow fiber UF module is greatly influenced by the molecular weight dispersity and molecular shape rather than the monomer composition. These results encouraged us to challenge the synthesis of a globular protein-like polymer support by using G7 PAMAM as a starting material.

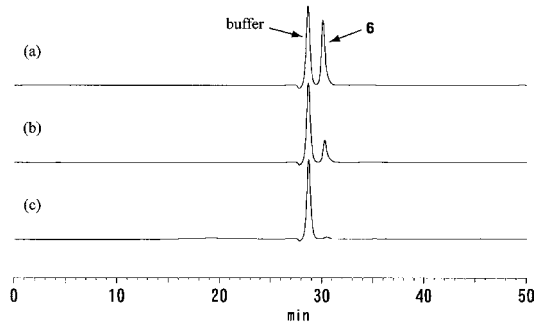

Figure 3. Capturing compound **6** by aminooxy-functionalized G7 PAMAM dendrimer **5**. (a) 0 h, (b) 8 h, and (c) 24 h. Elution condition: column, YMC-Pack Dial-200 (ϕ 8.0 × 500 mm); eluent, 50 mM sodium phosphate buffer, 0.3 M NaCl (pH 7.0); flow rate, 0.7 mL/min; column temperature, 25 °C; UV detector, 214 nm. Retention time for compound **6** is 30.1 min.

Automated Glycan Synthesis Using a Functional Dendrimer. To demonstrate the feasibility of dendrimers as an ideal polymer support in automated glycan synthesis, an aminooxy-functionalized dendrimer **5** was derived from the G7 PAMAM dendrimer by coupling with bis-Boc-aminooxyacetic acid succinimide ester[27] and deprotection. We selected 3-[*N*-(5-oxohexanoyl)-L-phenylanalyl-L-glutamyl-L-phenylalanyl-L-glycinyl]-aminopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**6**) as a test substrate modified with a heterobifunctional linker having a reactive ketone and the tetrapeptide moiety, Phe-Glu-Phe-Gly, as a specific cleavage site by *Bacillus licheniformis* BLase.[23] In the present study, we examined the synthesis of a sialyl Lewis X derivative **8** as a target compound due to its various significant biological functions and its associated difficulty in chemical synthesis in terms of the control of stereo- and regioselective glycosylation accompanying with multistep protection/deprotection procedures.[6−8,28] Figure 2 indicates a straightforward synthetic route to this target from a key starting G7 poly(amidoamine) dendrimer. After capturing the shuttle **6** released from solid-phase synthetic platform by an aminooxy-functionalized dendrimer **5**, we designed a fully automated multistep sugar elongation by three glycosyltransferases followed by selective release of the final product by treating the dendrimer with BLase.

As expected, HPLC monitoring revealed that shuttle molecule **6** (2.5 μmol) was quantitatively captured by employing 10 equiv mol of dendrimer **5**, in which 42 of 421 aminooxy-groups were theoretically consumed for the immobilization to afford primer **7** (Figure 3). G7 PAMAM dendrimers bearing shuttle **6** exhibited an excellent solubility and low viscosity as compared with a starting material, suggesting that an aminooxy-functionalized G7 PAMAM dendrimer may become a versatile scaffold for immobilizing relatively hydrophobic glycan derivatives and glycosphingolipids as well as various glycans and glycopeptides.

Conditions and details of a programmed operation for the fully automated synthesis of compound **8** from primer **7** on the Golgi apparatus are summarized in Figure 4. Three separated domains of the autosampler of Golgi were assigned as: (1) a

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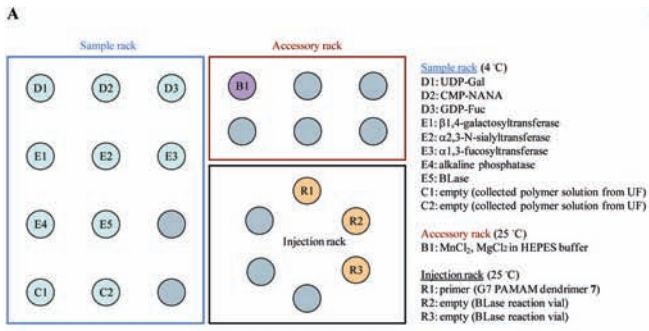

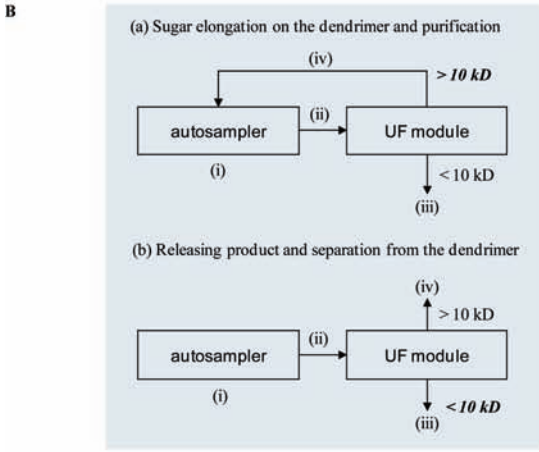

C

Function	Step	Command
Reaction	1	transfer $MnCl_2$ and MgCl (B1) to a primer (R1)
	2	transfer UDP-Gal (D1) to a primer (R1)
	3	transfer CMP-NANA (D2) to a primer (R1)
	4	transfer β1,4-galactosyltransferase (E1) to a primer (R1)
	5	transfer α2,3-N-sialyltransferase (E2) to a primer (R1)
	6	transfer alkaline phosphatase (E5) to a primer (R1)
	7	mix solution
	8	wait for 24 h (sialyl LacNAc synthesis on the dendrimer)
	9	transfer GDP-Fuc (D3) to a primer (R1)
	10	transfer α1,3-fucosyltransferase (E3) to a primer (R1)
	11	mix solution
	12	wait for 24 h (sialyl Lewis X synthesis on the dendrimer)
Separation	13	switch a UF flow path to permeate side
	14	inject reaction mixuture (R1) to a UF module
	15	purge permeate to waste
	16	exchange buffer from HEPES to ammonium acetate
	17	switch a UF flow path to retentate side
	18	recover retentate (product) to empty vials (C1, C2)
Reaction	19	transfer retentate (C1, C2) to empty vials (R2, R3)
	20	transfer BLase (E4) to solution containing dendrimer (R2, R3)
	21	mix solution
	22	wait for 24 h (release **8** from dendrimer)
Separation	23	switch a UF flow path to permeate side
	24	inject reaction mixuture (R2, R3) to a UF module
	25	recover permeate (containing **8**) to fraction collector
	26	switch a UF flow path to retentate side
	27	purge retentate to waste

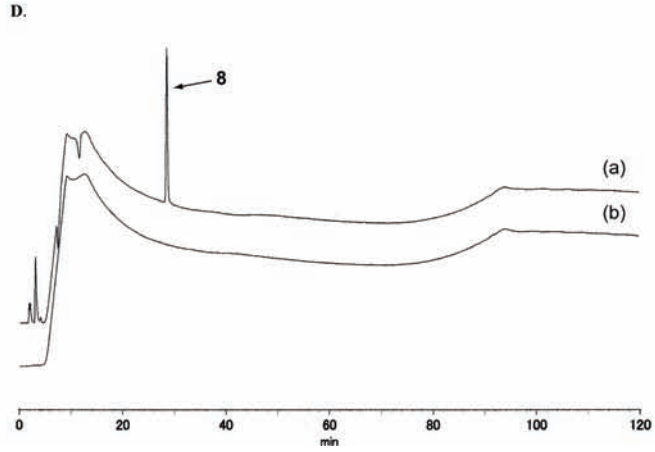

Figure 4. A programmed operation of automated synthesizer Golgi for the synthesis of sialyl Lewis X derivative **8** from primer **7**. (A) Details of a programmable autosampler in Golgi composed of three areas such as sample rack at 4 °C, accessory rack, and injection rack. (B) Schematic diagrams for the separation using UF module during sugar elongation reactions on the dendrimer and purification (a) as well as product release and separation from dendrimer (b), respectively. Functions of the individual steps are as follows: (i) multiple glycosylations by mixing glycosyltransferases, sugar nucleotides, alkaline phosphatase, and a primer solution; (ii) transferring a reaction mixture to the UF module; (iii) removing all small molecules and exchanging buffer solution from HEPES to ammonium acetate in (a) or collecting product **8** as a low molecular weight fraction in (b) (<10 kDa); (iv) collecting a retained dendrimer-supported sugar derivative (>10 kDa) in (a) or removing the retained dendrimer as a high molecular weight byproduct (>10 kDa) and cleaning up in (b). (C) Summary of automated glycan synthesis operated by a programmable 27 steps procedure. (D) HPLC profiles of crude product **8** (a) and blank (b). Condition: column, Shodex Asahipak NH2P-50 4E (ϕ 4.6 × 500 mm); eluent, A, 25 mM ammonium acetate (pH 5.8), eluent B, 25 mM acetic acid in acetonitrile (eluent A gradient increased from 15% to 98% over 90 min and isocratic elution at 98% for another 30 min); column temperature, 25 °C; UV detector, 210 nm; flow rate, 1.0 mL min^{-1}. Retention time for compound **8** is 28.5 min.

sample rack for sugar nucleotides (D1−3), enzymes (E1−5), and reservoirs to collect the dendrimer fraction (C1−2); (2) an accessory rack for HEPES buffer (B1) and; (3) an injection rack for primer **7** (R1) and reservoirs to collect the final product and dendrimer (R2−3), respectively (Figure 4A). As indicated in Figure 4B, a UF module was used for the purification of high-molecular weight dendrimer fractions during enzymatic sugar elongations (a, steps 1−18 in Figure 4C) while target **8** released from the dendrimer should be recovered as a small molecular fraction passed through the same hollow fiber UF module (b, steps 19−27 in Figure 4C). The program is based on two cycles of sequential functions, *reaction and separation*, for sugar elongations of primer **7** by one-pot sequential reactions of three glycosyltransferases in combination with designated sugar nucleotides and alkaline phophatase (*reaction* steps 1−12) and for purification of the dendrimer carrying sialyl Lewis X

tetrasaccharide by UF module (*separation* steps 13−18). Furthermore, the following procedures are performed for releasing **8** from dendrimer by BLase (*reaction* steps 19−22) and for final purification of **8** by removing polymers such as dendrimer and BLase using UF module (*separation* steps 23−27). The present synthetic platform allowed for fully automated enzymatic synthesis via Golgi for sialyl Lewis X tetrasaccharide derivative **8** (432 μg) within 4 days in 16% overall yield from a simple *N*-acetyl-D-glucosamine derivative (2.2 mg of shuttle **6**). Surprisingly, the HPLC profile as well as NMR and HRMS analyses of the *crude product* obtained directly from automated synthesis showed a satisfactory purity of **8** (Figure 4D). This means that no byproducts such as mono-, di-, or trisaccharide derivatives were generated during the sequential glycosylation reactions under a programmed operation.

Conclusion

In summary, these results demonstrate that the use of globular protein-like high molecular-weight dendrimer resulted in highly efficient and practical synthesis using some different-types of mammalian recombinant glycosyltransferases in as fully automatable manner as possible. The significance of the present observations is both methodological and fundamental. On the other hand, our findings should expand the scope of enzyme-assisted automated synthesis to include a range of glycan derivatives as important tools and probes for biological research and leads for drug discovery. It is also considered that efforts for the improvements and downsizing of the present HPLC-based Golgi machine will enhance the potency of the dendrimer-based strategy and versatility of automated glycan synthesis in common biochemical and medical laboratories without the need for specialized techniques.[29,30] Considering a significant loss of the product due to physical properties of hollow fiber-based material, it seems likely that ultrafiltration of dendrimer-based intermediates by some filter- or membrane-type modules might be a potential alternative to improve overall yield. It should also be emphasized that use of stable recombinant glycosyltransferases immobilized on the surface of some solid supports greatly contribute to the establishment of totally ideal automated glycan synthesis in a recyclable manner.[31] Although several limitations still exist in glycan synthesis and additional research is required to further advance this field, the ease of acquiring defined glycan structures with high purity from a machine will definitely impact on our understanding of carbohydrates in biological systems and on the development of carbohydrate-based therapeutics. Unfortunately, no general methods are, at present, available for the preparation of a wide range of complex carbohydrates of biological importance. However, it is clear that integrating dendrimer-supported enzymatic synthesis and chemical synthetic approaches such as solid-phase synthesis[3,5] and one-pot multistep glycosylations using unified monosaccharide building blocks[32] should expand the repertoire of synthetic glycans and their non-natural analogues which can be made, as well as allow for the creation of high-throughput screening methods such as glycan microarrays.[33,34]

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Supporting Information Available: Experimental section, picture and profile of artificial Golgi, and supplemental data of structural analysis of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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