Sugar Nucleotide Regeneration Beads (Superbeads): A Versatile Tool for the Practical Synthesis of Oligosaccharides

Xi Chen, Jianwen Fang, Jianbo Zhang, Ziye Liu, Jun Shao, Przemyslaw Kowal, Peter Andreana, and Peng George Wang*

Department of Chemistry, Wayne State University Detroit, Michigan 48202

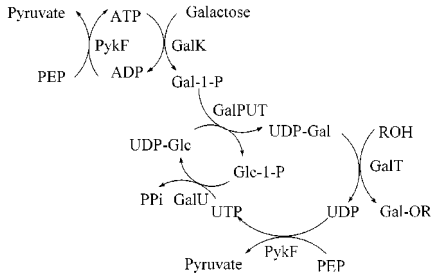
Received October 27, 2000

Application of carbohydrates in modern medicine is limited by the high cost of synthesis of most biologically important glycoconjugates. It is generally recognized that glycosyltransferase-catalyzed glycosylation is one of the most practical approaches.[1] Glycosyltransferases catalyze the transfer of a specific sugar from its sugar-nucleotide donor to an acceptor with high regio- and stereoselectivity. With increasing availability of recombinant glycosyltransferases in recent years, it can be expected that more researchers will use these enzymes to construct different glycoconjugates. The roadblock for practical use of this methodology is the high cost of necessary sugar nucleotides. So far the best solution is either using multiple-microbial fermentation systems based on patented microorganisms developed by Kyowa Hakko Kogyo Co. Ltd.[2] or using in vitro multiple-enzyme sugar nucleotide regeneration systems that avoid the need for stoichiometric amounts of sugar nucleotides. Such cycles were first demonstrated by Wong[3] and Whitesides and have been extensively developed by Wong and other groups to produce oligosaccharides.[4]

Here we report an approach that transfers in vitro multiple enzyme sugar nucleotide regeneration systems onto solid beads (the superbeads) which can be used and reused as common synthetic reagents for production of glycoconjugates.[5]

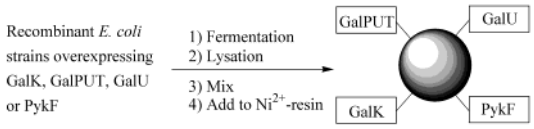
The preparation of such sugar nucleotide regeneration beads involves (i) cloning and overexpression of individual N-terminal His_6-tagged enzymes along the sugar nucleotide biosynthetic pathway and (ii) co-immobilizing these enzymes onto nickel-nitrilotriacetate (NTA) beads.[6] The sugar nucleotide regeneration superbeads can then be conveniently combined with glycosyltransferases for specific oligosaccharide sequences.

Scheme 1. Biosynthetic Pathway for Galactosides with Regeneration of UDP-Gal[a]



[a] Abbreviations: PEP, phosphoenolpyruvate; Gal-1-P, galactose-1-phosphate; Glc-1-P, glucose-1-phosphate; PPi, pyrophosphate.

Scheme 2. UDP-Gal Regeneration Beads



The first generation of superbeads we developed is for UDP-Gal regeneration. As shown in Scheme 1, the regeneration of UDP-Gal from UDP (byproduct of the galactosylation) requires four enzymes: galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalPUT), glucose-1-phosphate uridylyltransferase (GalU), and pyruvate kinase (PykF). Thus, corresponding *galK*, *galT*, *galU*, and *pykF* genes were individually amplified from *E. coli* K-12 genome by polymerase chain reaction, and then inserted into the pET15b vector with a sequence coding for a N-terminal His_6-tag, respectively. The enzymes were expressed in *E. coli* BL21 (DE3) with isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction. Cell lysate mixture with an equal activity of individual enzymes was prepared by combining the cell lysate (in 20 mM Tris-HCl, pH 8.5 buffer containing 1% Triton X-100, 200 μg/mL of lysozyme, and 2 μg/mL of Dnase I) with a relative volume ratio of GalK:GalPUT:GalU:PkyF = 4:1:1:2.[7] The UDP-Gal regeneration beads were obtained by incubating the cell lysate mixture with Ni^{2+}-NTA resins (3 mL of lysate mixture mL of beads) for 20 min and washing with a Tris-HCl (20 mM, pH 8.0) containing 0.5 M NaCl (Scheme 2). Enzymatic assays indicated that each enzyme was quantitatively immobilized onto the beads with 1.5 U of each immobilized enzyme per milliliter of beads.

The application of UDP-Gal regeneration beads was demonstrated by the production of Galα1,3Galβ1,4GlcOBn **1** (Table 1, entries 1 and 2) with a truncated bovine α-1,3-galactosyltransferase (α1,3GalT) expressed in *E. coli*.[8] Oligosaccharides with a terminal Galα1,3Gal sequence (α-Gal epitopes) are desirable as antigens for preventing hyperacute rejection in pig-to-human xenotransplantation.[9] In the gram-scale synthesis, the superbeads (40 mL, containing 60 U of GalK, GalPUT, GalU, and PykF) were incubated with a cell lysate of α1,3GalT (40 mL, 40 U, quantitative immobilization), washed by Tris-HCl buffer (20 mM, pH 8.0) containing 0.5 M NaCl, and added to a reaction mixture of LacOBn (1 g, 2.4 mmol), ATP (132 mg, 240 μmol), PEP (912 mg, 4.8 mmol), UDP (100 mg, 240 μmol), Glc-1-P (73 mg, 240 μmol), Gal (540 mg, 3 mmol), $MgCl_2$ (10 mM), $MnCl_2$ (10 mM), and KCl (100 mM) in HEPES buffer (100 mM, pH 7.5) to a total volume of 250 mL. The reaction was stirred at room

* To whom correspondence should be addressed. Phone: 313-993-6759. Fax: 313-577-2554. E-mail: pwang@chem.wayne.edu.

(1) (a) Wymer, N.; Toone, E. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 110. (b) Palcic, M. M. *Curr. Opin. Biotechnol.* **1999**, *10*, 616. (c) Crout, D. H. G.; Vic, G. *Curr. Opin. Chem. Biol.* **1998**, *2*, 98.

(2) (a) Tabata, K.; Koizumi, S.; Endo, T.; Ozaki, A. *Biotech. Lett.* **2000**, *22*, 479. (b) Endo, T.; Koizumi, S.; Tabata, K.; Ozaki, A. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 257. (c) Endo, T.; Koizumi, S.; Tabata, K.; Kakita, S.; Ozaki, A. *Carbohydr. Res.* **1999**, *316*, 179. (d) Koizumi, S.; Endo, T.; Tabata, K.; Ozaki, A. *Nature Biotechnol.* **1998**, *16*, 847.

(3) Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5418.

(4) (a) Gilbert, M.; Bayer, R.; Cunningham, A. M.; DeFrees, S.; Gao, Y.; Watson, D. C.; Young, N. M.; Wakarchuk, W. W. *Nat. Biotechnol.* **1998**, *16*, 769. (b) Noguchi, T.; Shiba, T. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 1594. (c) Zervosen, A.; Elling, L. *J. Am. Chem. Soc.* **1996**, *118*, 1836. (d) Look, G. C.; Ichikawa, Y.; Shen, G.-J.; Cheng, G.-J.; Wong, C.-H. *J. Org. Chem.* **1993**, *58*, 4326. (e) Wang, P.; Shen, G.-J.; Wang, Y.-F.; Ichikawa, Y.; Wong, C.-H. *J. Org. Chem.* **1993**, *58*, 3985. (f) Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283. (g) Wong, C.-H.; Wang, R.; Ichikawa, Y. *J. Org. Chem.* **1992**, *57*, 4343. (h) Gygax, D.; Spies, P.; Winkler, T.; Pfarr, U. *Tetrahedron* **1991**, *28*, 5119. (i) Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698.

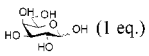
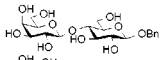
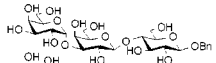
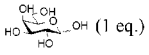
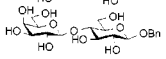
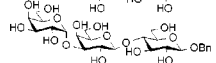
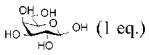
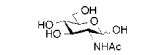
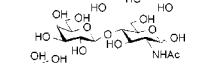
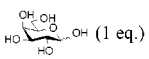
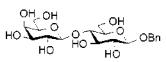
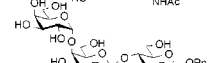
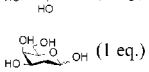
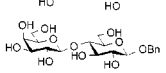
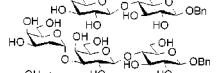
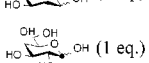
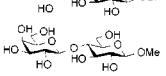
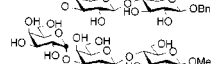
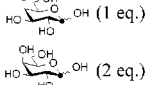
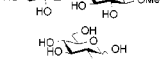
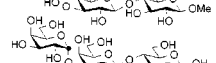
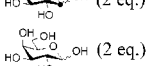
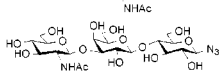
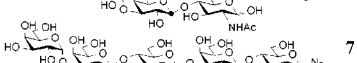
(5) Representative references on using immobilized enzymes in carbohydrate synthesis: (a) Fujita, K.; Tanaka, N.; Sano, M.; Kato, I.; Asada, Y.; Takegawa, K. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 134. (b) Revers, L.; Bill, R. M.; Wilson, I. B.; Watt, G. M.; Flitsch, S. L. *Biochim. Biophys. Acta* **1999**, *1428*, 88. (c) Lubineau, A.; Basset-Carpentier, K.; Auge C. *Carbohydr. Res.* **1997**, *300*, 161. (d) Asano, N.; Oseki, K.; Kaneko, E.; Matsui, K. *Carbohydr. Res.* **1994**, *258*, 255. (e) Roth, S. U.S. Patent 5 583 042, 1996. (f) Roth, S. U.S. Patent 5 288 637, 1994. (g) Roth, S. U.S. Patent 1 580 674, 1993.

(6) Ni^{2+}-NTA agarose was from Qiagen, Santa Clarita, CA. Bead size, 45−165 mm; bead structure, cross-linked 6% agarose; support, sepharose CL-6B; protein capacity, 300−500 nmol/mL.

(7) The activities and specific activities of recombinant enzymes in the cell lysate (25 mL of lysate per 1 L of cell culture) were 25 U/L and 1 U/mg, 100 U/L and 4 U/mg, 100 U/L and 6 U/mg, and 50 U/L and 1 U/mg for GalK, GalPUT, GalU, and PykF, respectively. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of product per minute at 24 °C. The amounts of the enzymes were determined by the Lowry method (Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265) after purification.

10.1021/ja005738v CCC: $20.00
Published on Web 02/08/2001

Table 1. Preparative Syntheses of Oligosaccharides with UDP-Gal Regeneration Superbeads

Entry	GalTs	Starting Gal	Acceptor	Products		Yields (%)
1	α1,3GalT on bead	 (1 eq.)			**1**	85 (72[a])
2	α1,3GalT in solution	 (1 eq.)			**1**	78
3	β1,4GalT in solution	 (1 eq.)			**2**	92
4	α1,4GalT on bead	 (1 eq.)			**3**	86
5	α1,3GalT on bead	 (1 eq.)			**4**	69
6	α1,3GalT on bead	 (1 eq.)			**5**	83
7	α1,3GalT on bead β1,4GalT on bead	 (2 eq.)			**6**	95
8	α1,3GalT on bead β1,4GalT on bead	 (2 eq.)			**7**	76

[a] Gram-scale synthesis. Others are 100 mg-scale reactions.

temperature (24 °C) for 4 days. When thin-layer chromatographic (TLC) analysis [*i*-PrOH:NH_4OH:H_2O = 7:3:2 (v/v/v)] indicated that the reaction was complete, the superbeads were separated from the reaction mixture by centrifugation and washed for another reaction. The product was purified from reaction mixture by a Sephadex G-15 gel filtration column with water as the mobile phase. The trisaccharide-containing fractions were pooled and lyophilized to give Galα1,3LacOBn **1** (1.03 g, 72% yield based on acceptor LacOBn). The beads were then reused three times (yields were 71%, 69%, and 66%, respectively) during a three-week period, retaining 90% enzyme activity based on α1,3GalT. After several repeated syntheses, the deactivated enzymes were removed from the nickel beads, and the beads were recharged for more uses. It was observed that the effect of using inorganic pyrophosphatase to remove the inhibition of pyrophosphate to the glycosylation was minimal under current reaction conditions.[2d] Thus, addition of the pyrophosphatase was omitted.

The galactosyltransferases can also be used in solution. For entry 2, the N-terminal His-tag of the recombinant α1,3GalT was cleaved by treatment with thrombin for 16 h and removed by dialysis against Tris-HCl buffer (20 mM, pH 7.9) containing 10% glycerol. The enzyme solution was then added to the superbead reaction mixture to synthesize Galα1,3LacOBn **1**; a yield of 78% was achieved (Table 1, entry 2).

The versatility of the UDP-Gal regeneration beads was demonstrated in the synthesis of a variety of oligosaccharides (Table 1, entries 3 and 4). A combination of the beads with bovine *β*-1,4-galactosyltransferase (from Sigma) in solution readily produced Gal*β*1,4GlcNAc **2** in 92% yield (Table 1, entry 3). Combination of the beads with a recombinant α-1,4-galactosyltransferase cloned from *Neisseria meningitidis* (Kowal and Wang, unpublished data) on beads (100% immobilization) produced Galα1,4Gal*β*1,-4GlcOBn **3** (Table 1, entry 4) in 86% yield. This sugar sequence (called globotriose Gb_3) is a trisaccharide portion of globotriaosylceramide. Synthetic Gb_3 derivatives could be effective inhibitors of globotriaosylceramide-verotoxin interaction.[10]

Another powerful synthetic potential of the superbeads is that the beads can also take some unnatural monosaccharides as starting materials to synthesize unnatural oligosaccharides. For example, when 2-deoxygalactose was used as the starting monosaccharide instead of galactose, a combination of the UDP-Gal regeneration beads with on-beads α1,3GalT generated a novel 2-deoxy α-Gal epitope **4** (Table 1, entry 5). Similarly, the use of 1-^{13}C-labeled galactose generated 1-^{13}C-labeled α-Gal epitope **5** (Table 1, entry 6).

The UDP-Gal regeneration beads can be used in combination with multiple galactosyltransferases. For example, both α1,3GalT and *β*1,4GalT[11] can be simultaneously immobilized onto the beads to generate specific Galα1,3Gal*β*1,4Glc sequence producing beads. Using 2 equiv of 1-^{13}C-labeled galactose and 4 equiv of PEP as starting materials, double 1-^{13}C-labeled trisaccharide **6** was produced (Table 1, entry 7) from GlcNAc when enough reaction time (10 days) was given. Disaccharide (1-^{13}C)-Gal*β*1,4GlcNAc was formed as an intermediate as indicated by TLC during the reaction process. Similarly, using 2 equiv of galactose as starting sugar and GlcNAc*β*1,3Gal*β*1,3GlcN_3 as an acceptor, pentasaccharide **7** (Table 1, entry 8) was produced with an overall yield of 76%. Both 1-^{13}C-labeled α-Gal epitopes (**5** and **6**) are being used in NMR experiments to determine the solution conformation of α-Gal epitopes and their interaction with anti-Gal antibody.

In summary, we have demonstrated that UDP-Gal regeneration beads can be generated by an amalgamation of recombinant enzymes along the biosynthetic pathway of UDP-Gal. When combined with galactosyltransferase(s) either on beads or in solution, the beads can be used as a common reagent to synthesize a variety of oligosaccharides and their derivatives. Such beads inherit all the advantages of enzyme immobilization and cell free solid-phase organic synthesis such as ease of separation, increased stability, reusability, and improved kinetics.[12] Work is in progress to make other common sugar-nucleotide regeneration beads. It can be envisioned that these will become a new generation of bio-reagents that can be coupled with a variety of glycosyltransferases for the production of glycoconjugates and their derivatives.

Supporting Information Available: Cloning, expression, purification, and assay procedures for GalK, GalPUT, GalU and PykF; procedures to prepare UDP-Gal regeneration beads and their combination with galactosyltransferase(s); synthetic procedures, and spectral data for compounds **1**−**7** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA005738V

(8) (a) Chen, X.; Zhang, W.; Wang J. Q.; Fang, J. W.; Wang, P. G. *Biotechnol. Prog.* **2000**, *16*, 595. (b) Fang, J. W.; Li, J.; Chen, X.; Zhang, Y. N.; Wang, J. Q.; Guo, Z. M.; Zhang, W.; Yu, L. B.; Brew, K.; Wang, P. G. *J. Am. Chem. Soc.* **1998**, *120*, 6635.

(9) Chen, X.; Andreana, P. R.; Wang, P. G. *Curr. Opin. Chem. Biol.* **1999,** *3,* 650.

(10) (a) Peter, M. G.; Lingwood, C. A. *Biochim. Biophys. Acta* **2000**, *1501*, 116. (b) Barnett, F. D.; Abul-Milh, M.; Huesca, M.; Lingwood, C. A. *Infect. Immun.* **2000**, *68*, 3108. (c) Lingwood, C. A. *Biosci. Rep.* **1999**, *19*, 345. (d) Lingwood, C. A. *Biochim. Biophys. Acta* **1999**, *1455*, 375.

(11) Zhang, Y.; Malinovskii, V. A.; Fiedler, T. J.; Brew, K. *Glycobiology* **1999**, *9*, 815.

(12) (a) Clair, N. L. S.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314. (b) Thiem, J.; Wiemann, T. *Synthesis-Stuttgart* **1992**, *1*−*2*, 141. (c) Bulow, L.; Mosbach, K. *Trends Biotechnol.* **1991**, *9*, 226. (d) Bulow, L. *Eur. J. Biochem.* **1987**, *163*, 443. (e) Auge, C.; Mathieu, C.; Merienne, C. *Carbohydr. Res.* **1986**, *151*, 147.