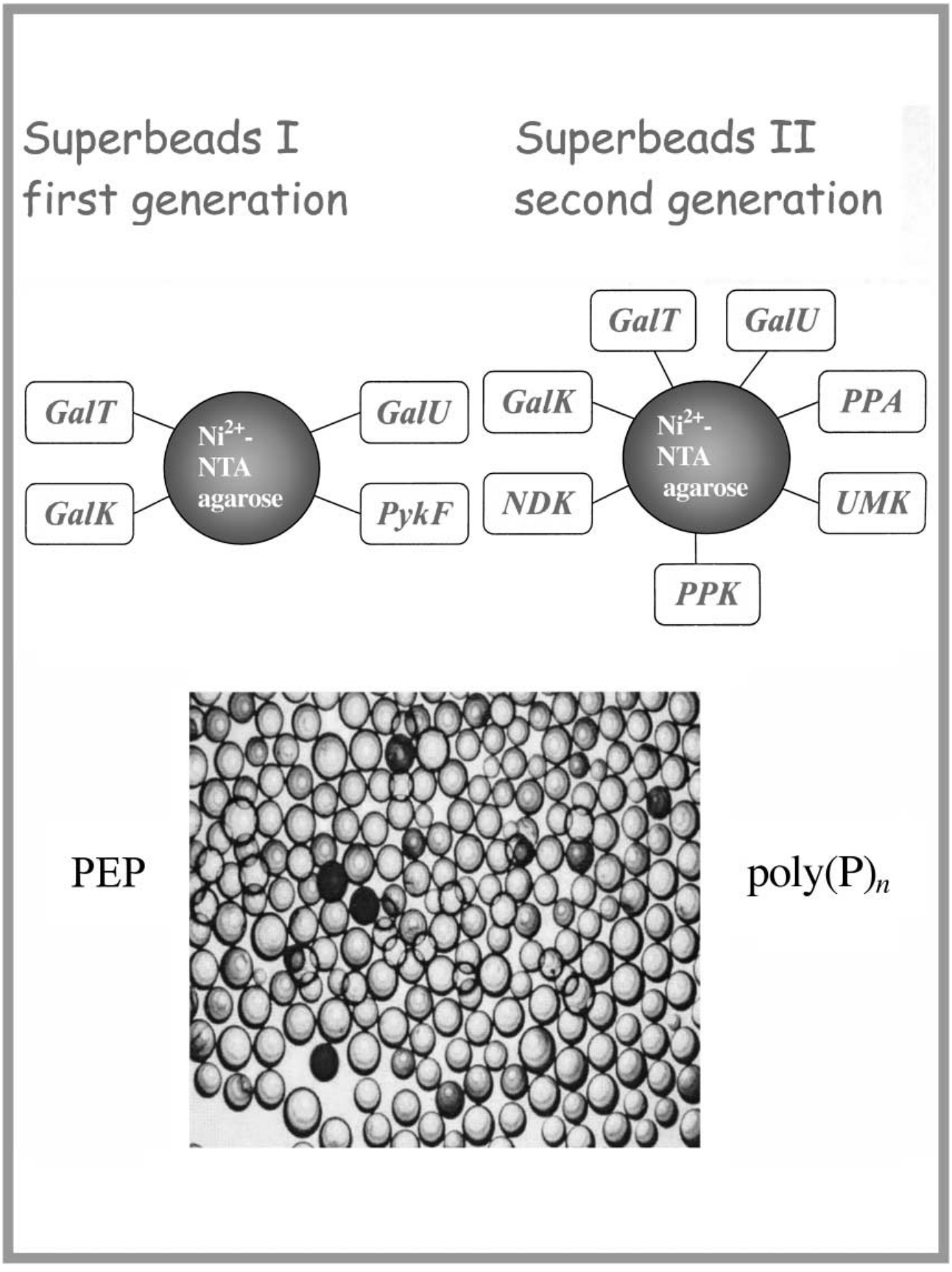

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Superbeads: Immobilization in "Sweet" Chemistry

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Abstract: Enzymatic oligosaccharide synthesis using recombinant glycosyltransferases is able to overcome the difficulties associated with chemical methods. Nonetheless, sugar nucleotide regeneration cycles are necessary for the glycosylation. The multistep enzyme reaction can be efficiently carried out on superbeads that are prepared by immobilizing multienzyme mixtures on bead support through fused binding domains.

Keywords: enzyme catalysis · immobilization · oligosaccharides · sugar nucleotide regeneration

Introduction

Glycobiotechnology is leading to new products such as glycoconjugates and polysaccharides that are potential pharmaceuticals and components in drug design. Many sugar-based drugs are known but they are sparsely used in the pharmaceutical industry mainly due to the immature production methods. One of the main reasons for this stems from the inability to produce sufficient quantities of the required oligosaccharides at a reasonable price. Thus it remains a challenge for synthetic carbohydrate chemists to provide ever more potent strategies. Generally, it is recognized that enzyme catalyzed glycosylation is one of the most practical approach for large-scale synthesis.[1] A number of enzymatic methods have been developed so far:

1) Oligosaccharide synthesis using glycosyltransferases of the Leloir biosynthetic pathway, which require sugar nucleotides as donors,
2) Oligosaccharide synthesis using glycosyltransferases of the non-Leloir biosynthetic pathway, which require sugar 1-phosphates as donors,
3) Oligosaccharide synthesis using glycosidases.

The use of Leloir glycosyltransferases seems to be the most advantageous method for large-scale production because of the high regioselectivity and yields. Figure 1 shows the Leroil glycosyltransferase synthetic procedure.

Recombinant technologies are now providing the required glycosyltransferases (step III, Figure 1); additionally the accumulation of new data on their genes, structures and mechanisms has increased the pace in recent years.[2] On the other hand, required sugar nucleotides (NDP-sugars, step II, Figure 1) are not commercially available in large quantity, and also nucleoside 5′-triphosphates (NTP, step I, Figure 1), in contrast to monophosphates (NMP), are relatively expensive. Therefore, many studies are conducted to develop techniques for NTP and NDP-sugars (re)generation. The sources of energy for regeneration consist of cheaper high-energy phosphate compounds or polyphosphate (step I, Figure 1).[3] Polyphosphate is the cheapest alternative. A combination of NMP kinases with polyphosphate kinase (ppk)[4] or polyphosphate/NMP phosphotransferase with ppk[5] makes ppk and polyphosphate the most attractive method for NTP and NDP-sugars (re)generation.

If the above-mentioned enzyme approach is to be used in an economically feasible industrial process, enzyme recycling is essential. Generally, recycling of any biocatalyst (enzymes, cells, ribozymes, abzymes and multicatalytic complexes) is most convenient using immobilization. Various immobilization strategies have been devised,[6] however, there are no general rules for selecting the best approach for a given application.

In the eighties, Professor Serge David and Claudine Augé started a project on the use of immobilized enzymes in preparative sugar chemistry.[7] In 2000 year, Augé and co-workers introduced to the field an efficient procedure for concentration, immobilization and stabilization of fucosyltransferase, relying on a His_6 tag of the recombinant enzyme.[8] We reported a transfer of all in vitro multiple enzyme sugar nucleotide regeneration systems onto a support (superbeads).[9] The concept of such sugar nucleotide regeneration beads involves: i) cloning and overexpression of N-terminal His_6-tagged enzymes along the sugar nucleotide biosynthetic pathway and ii) co-immobilization of these enzymes onto nickel-nitrilotriacetate (NTA) agarose.

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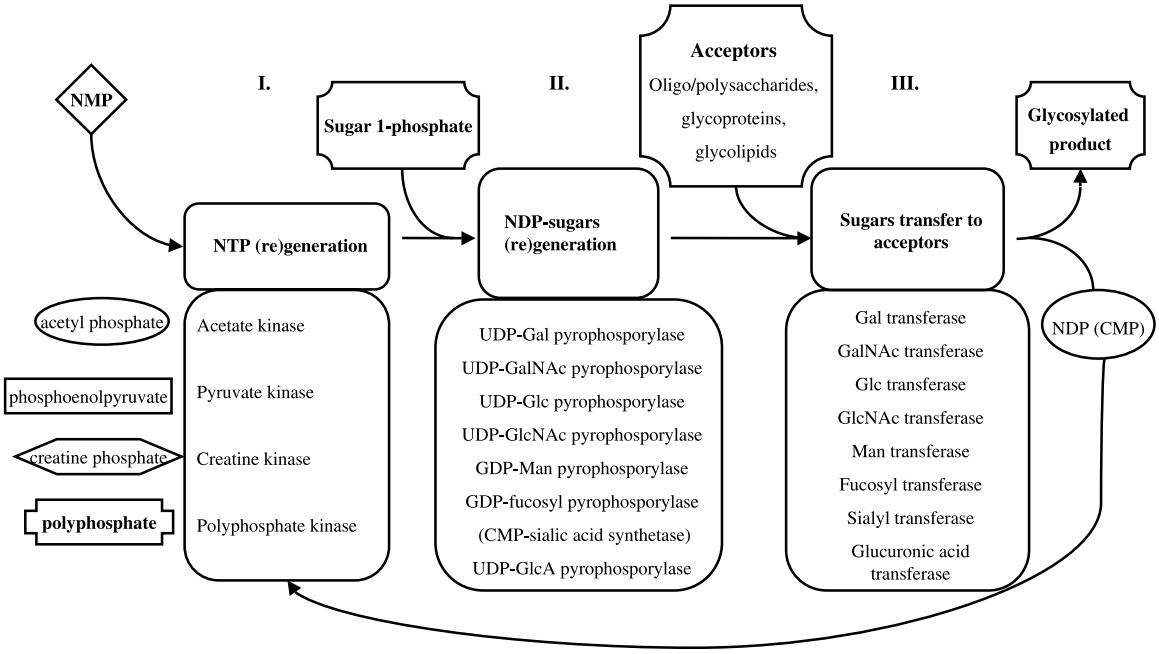
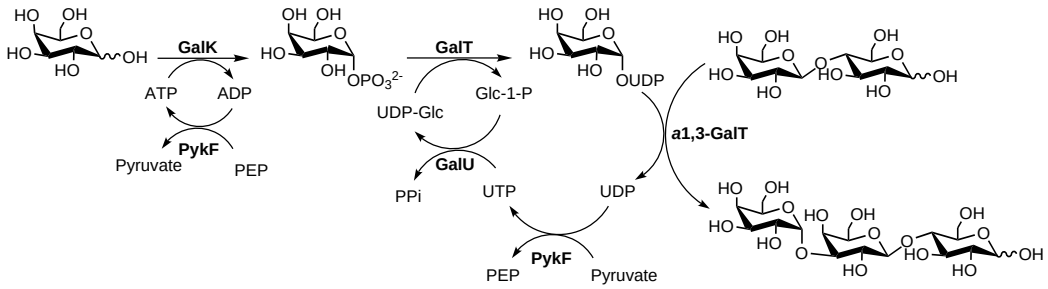


Figure 1. Oligosaccharide synthesis by Leloir glycosyltransferases.

Enzymes along the sugar nucleotide biosynthetic pathways: Biochemical pathway charts reveal many enzymes for the synthesis of nucleotide sugars. The majority of them are available as recombinant proteins; however, only a few of them have been characterized in terms of suitability for the development of industrial biocatalytic processes. In this respect, the enzymes from microbial sources are suited much better for nucleotide sugar synthesis despite the fact that transferases from mammalian sources have been available for some time. There are eight main sugar nucleotides which are the substrates of the Leloir glycosytransferases in mammals (Figure 1). Their enzymatic syntheses have been well summarized by Bülter and Elling.[10] Uridine 5′-diphospho-α-D-galactose (UDP-Gal) is one of the most relevant nucleotide sugars to glycoconjugate biosynthesis; we therefore focused on its large-scale preparation. Two generations of superbeads have been developed (see also Frontispiece):[9] superbeads I and superbeads II. The first generation requires four enzymes (Scheme 1) and second generation seven enzymes (Scheme 2) for UDP-Gal (re)generation. The corresponding genes were individually amplified from *E. coli* K-12 genome by polymerase chain reaction, and then inserted into pET15b vector with a sequence coding for a N-terminal His_6-tag. The enzymes were expressed in *E. coli* BL21(DE3) with isopropyl-1-thio-β-galactopyranoside (IPTG) induction. Cell lysate mixtures with an equal activity of individual enzymes were prepared by combining the cell lysates (in 20 mM Tris-HCl, pH 8.5 buffer containing 1% Triton X-100) with the appropriate volume ratios.

Immobilization of the enzymes: The use of immobilized enzymes in bioprocesses offers greater productivity because the same enzyme molecules can be used over a long period of time. Other advantages include more precise control of the extent of the reaction, the capability of automation and continuous operation, and the elimination of the requirement for downstream enzyme inactivation. These reasons propelled the development of many immobilization methods such as: covalent binding, encapsulation, entrapment, cross-linking and adsorption.[6] Covalent binding[11] avoids the disadvantage of basic adsorption methods, that is desorption of enzymes under environmental changes (pH, temperature) or shaking of the biocatalyst–support complex. However, desorption is turned to an advantage if the regeneration of the support is built into operational regimen to allow rapid expulsion of exhausted biocatalyst and replacement with fresh enzymes.[12]



Scheme 1. Biosynthetic pathway for UDP-Gal regeneration and α-Gal production. Galactokinase (GalK, EC2.7.1.6), galactose-1-phosphate uridylyltransferase (GalT, EC2.7.7.12), UDP-glucose pyrophosphorylase (GalU, EC2.7.7.9), and pyruvate kinase (PykF, EC2.7.1.40).

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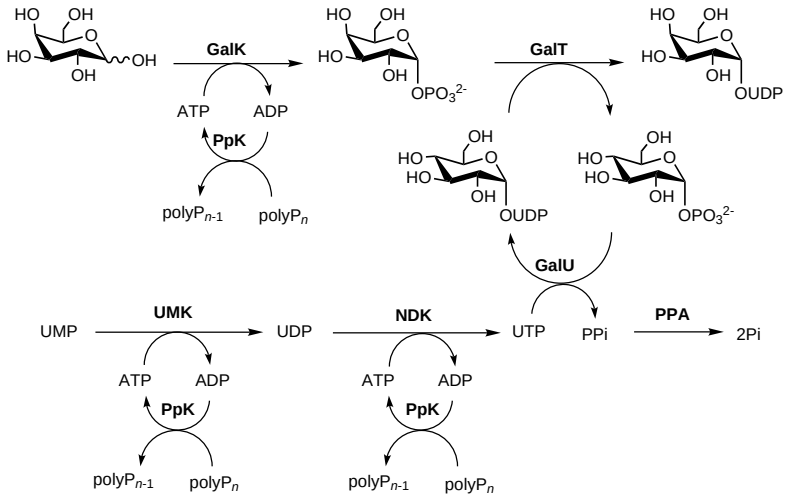

Scheme 2. Biosynthetic pathway for UDP-Gal regeneration. Galactokinase (GalK, EC2.7.1.6), galactose-1-phosphate uridyltransferase (GalT, EC2.7.7.12), UDP-glucose pyrophosphorylase (GalU, EC2.7.7.9), inorganic pyrophosphatase (PPA, EC3.6.1.1), UMP kinase (UMK, EC2.7.4.14), nucleotide diphosphate kinase (NDK, EC2.7.4.6), polyphosphate kinase (PpK, EC2.7.4.1).

Encapsulation of the enzyme with nylon or cellulose nitrate semipermeable membranes causes diffusion problems which may result in the rupture of the membrane if the products rapidly accumulate from the reaction mixture. Entrapment of the enzyme into a three-dimensional matrix of native (alginate, agarose) or synthetic (polyvinylalcohol) hydrogels decreases the diffusional limitations. However, in contrast to encapsulation, the gel lattice is not tight enough to prevent enzyme leakage and thus requires an increase in the size of catalytic particles through aggregation[13] or coating of the gel bead surface with additional polymers.[14] Immobilization of enzymes by adsorption on solid support has been pursued since the 1950s and is the method used the most in industrial biocatalysis. The procedure consists of combining the biological component(s) and support with adsorption properties, under suitable conditions such as pH, ionic strength for an incubation period, followed by collecting the immobilized material and extensive washing to remove nonbound biological component. Surface modified silicon, silica gel, glass or native cellulose, agarose, gelatin and chitosan are examples of such supports. Integration of immobilized metal-affinity chromatography (IMAC)[15] and bioaffinity chromatography[16] in the design of enzyme expression and adsorption solved nonspecific binding on the support, lowered the cost and made this approach superior from the industrial standpoint. Many hybrid proteins were prepared by fusing the coding sequence of enzyme (functional domain) and the coding sequence for an affinity peptide (binding domain). As mentioned above, we have used enzymes along the sugar nucleotide biosynthesis pathways (Schemes 1 and 2) with fused hexahistidine tail. The attachment of recombinant enzymes to Ni^{2+}-agarose beads is shown in Figure 2. Gal regeneration beads were prepared by incubating the cell lysate mixtures with Ni^{2+}-NTA resin from Qiagen (3 mL lysate, 1 mL beads) for 20 min and washings with Tris-HCl (20 mM, pH 8.0) buffer containing 0.5 M NaCl.

Other, most powerful strategy relies on the cellulose-binding domain (CBD) which is a peptide derived from the cellulolitic bacteria such as *Cellulomonas fimi*[16] or *Clostridium cellulovorans* (cellulose binding protein A).[17] CBD shows very strong affinity for cellulose and chitin.[18] Its binding to cellulose is essentially irreversible under high-salt concentration and wide range of pH 2–10. Only 6 M guanidine-HCl, 8 M urea or high pH can remove CBD from cellulose. Cellulose and chitin beads are relatively inexpensive, chemically inert materials, which are safe for use even in food or pharmaceutical applications. Many native immobilization matrices with different properties are available. Consequently, it is anticipated that

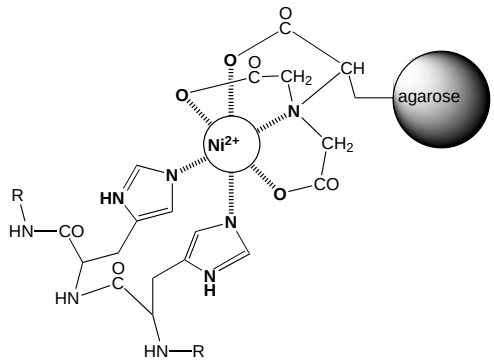

Figure 2. Attachment of recombinant enzymes to Ni^{2+}-agarose by the histidine tail.

other support binding domains (e.g. xylan-binding domain,[19] starch-binding domain[20]) will likely be used in the near future and offer adsorption tailored to a selected immobilization method (e.g. alginate entrapment/alginate-binding protein[21]). Therefore it follows to offer a hypothesis that multiple (bio)affinity layering[22] first established between lectins and saccharides can also improve the immobilization by "support" binding domains of recombinant proteins (Figure 3).

Application of the superbeads: The application of UDP-Gal regeneration beads was demonstrated by the production of a variety of oligosaccharides (Figure 4). The synthesis of Galα1,3Galβ1,4GlcOBn (**1**), trisaccharide with a terminal Galα1,3Gal sequence (αGal-epitope, desirable as antigen for preventing hyper-acute rejection in pig-to-human xenotransplantation[23]) has been performed in gram-scale. When *E. coli* lysate containing bovine α-1,3-galactosyltransferase was added to the enzyme mixture (Scheme 1) and co-immobilized on Ni^{2+}-agarose support, 72% yield based on LacOBn acceptor has been obtained. The repeated use of superbeads gave 71,

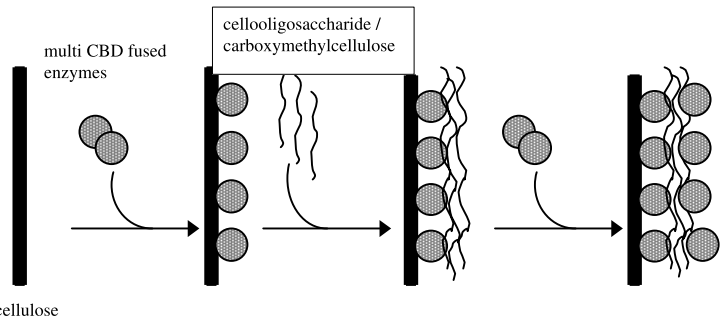

Figure 3. Schematic diagram of the bioaffinity layers preparation.

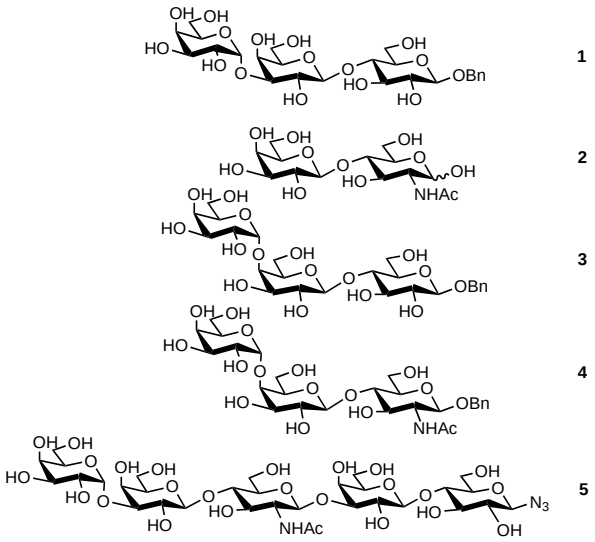

Figure 4. Oligosaccharides synthesized with UDP-Gal regeneration beads.

69, and 66% yields during a three-week period. The same galactosyltransferase (with removed His_6-tag) used in solution of the reaction mixture gave the yield of 78%. A combination of the beads with bovine β-1,4-galactosyltransferase (from Sigma) in solution readily produced Galβ1,4GlcNAc (**2**) in 92% yield based on GlcNAc. Globotriose (**3**) a trisaccharide inhibitor of globotriaosylceramide/verotoxin interaction[24] was produced by the recombinant α-1,4-galactosyltransferase from *Neisseria meningitidis* co-immobilized on superbeads I in 86% yield. Another synthetic potential of the superbeads is that multiple galactosyltransferases can be simultaneously immobilized onto the beads to generate specific sequences. For example, the combination of α-1,3-galactosyltransferase with β-1,4-galactosyltransferase produced Galα1,3Galβ1,4Glc (**4**) in 95% and Galα1,3Galβ1,4GlcNAcβ1,3Galβ1,3GlcN_3 (**5**) in 76% yield.

The reactions were performed in repeated batch mode with stirred reactor or packed column bed reactor configurations.[9] After several repeated syntheses, the deactivated enzymes can be removed from the nickel beads by washing with 0.5 M EDTA; the resin is then recharged with a $NiCl_2$ solution and new enzyme mixture.[25]

Oligosaccharide synthesis using whole cells: An alternative strategy for the synthesis of oligosaccharides is the in vivo, intracellular production in recombinant microorganisms expressing glycosyltransferases and enzymes along sugar nucleotide synthetic pathways. This method has the advantage over classical chemoenzymatic methods that there is no need for any overproduction and pre-purification of the enzymes. The whole cell approach has grown in significance when we successfully transferred the complex in vitro biosynthetic cycle (Scheme 1) into a single, product-producing *E. coli* strain containing a plasmid with all the necessary genes for sugar-nucleotide (re)generation and oligosaccharide accumulation.[26] The carbohydrate-producing bacterial strains—we call "superbugs"—can be fermented in large quantities and directly used for the synthesis of the desired oligosaccharide. Without extensive optimization, the superbug system produces 3–4 g of Galα1,3Lac trisaccharide from every 10 L fermentation (about 56% yield). We should point out that the superbug synthesis is a two-step process; the first step involves the growth of the engineered microorganisms and the second step involves their use as a source of catalyst, energy, and cofactors to synthesize the product.

Another strategy, pioneered by Kyowa Hakko company in Japan, uses coupling of three strains: I) *Corynebacterium ammoniagenes* for NTP (re)generation; II) recombinant *E. coli* with NDP-sugar (re)generation cycle expression; and III) recombinant *E. coli* with glycosyltransferase. Despite the need for multiple plasmids, multiple fermentations of several strains, and transport of intermediates between the strains, this approach is the most cost effective at this time. The Kyowa Hakko group achieved large-scale production of globotriose (188 gL^{-1}), *N*-acetyllactosamine (107 gL^{-1}), sialyl-T_n epitope (45 gL^{-1}) and 3′-sialyllactose (33 gL^{-1}).[27]

Immobilization of the cells: The fact that whole cell immobilization has been applied in pharmaceutical biotransformations as an alternative to enzyme immobilization highlights its economic benefits. Not only are the costs for enzyme isolation eliminated but frequently, enzyme system in native cell preparations exhibits greater stability than in a purified state. However, a disadvantage of the whole cell system is side-product contamination and product degradation.

Because of the simplicity, nontoxicity, low cost, and versatility, entrapment of cells into calcium alginate has been established as basic cell immobilization method during last three decades. Recently, some companies (e.g. GeniaLab, Inotech AG, Nisco Engineering AG) have worked out scale up of the entrapment to alginate and introduced large-scale encapsulation apparatus.[28] Sometimes calcium alginate does not provide satisfactory results, because it is sensitive to calcium chelating compounds, especially to high levels of phosphate used in glycosyltransferase catalyzed synthesis of saccharides. In those cases, the alginate can simply be substituted with pectate[29] or with a synthetic hydrogel such as polyvinyl alcohol.[30]

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Although whole-cell immobilization via action of surface-expressed CBD has already been demonstrated,[31] the effectiveness and precise conditions for biotransformations have not been elucidated. The main problem is the low immobilization capacity and that binding is dramatically affected at higher pH. However, immobilization of whole cells by "support" binding domains is a very promising method for the future.

Despite the fact that Kyowa Hakko technology and our superbugs shifted oligosaccharide synthesis by recombinant glycosyltransferases to the whole cell reactions, there is no example for the immobilization of the latter.

Conclusion

Oligosaccharide synthetic chemistry is integrated with the modern strategy for pharmaceutical synthesis: The concept involves 1) deduction of the biosynthetic pathways of a desired product using genomic and protein databases; 2) cloning, expression, and investigation of the individual enzymes; 3) immobilization of the enzymes by fused binding domains or immobilization of whole recombinant cells which overexpress the synthetic cycle; and 4) running the reaction by robust immobilized preparation, that is superbeads. These four points require close collaboration between synthetic chemists, biochemist, as well as molecular biologists.

[1] X. Chen, P. Kowal, P. G. Wang, *Curr. Opin. Drug Discovery Dev.* **2000**, *3*, 756–763.

[2] a) U. M. Ünligil, J. M. Rini, *Curr. Opin. Struct. Biol.* **2000**, *10*, 510–517; b) T. Endo, S. Koizumi, *Curr. Opin. Struct. Biol.* **2000**, *10*, 536–541.

[3] a) Y. Ichikawa, R. Wang, C. H. Wong, *Methods Enzymol.* **1994**, *247*, 107–127; b) T. Noguchi, T. Shiba, *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1594–1596.

[4] K. Ishige, T. Hamamoto, T. Shiba, T. Noguchi, *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1736–1740.

[5] A. Kameda, T. Shiba, Y. Kawazoe, Y. Satoh, Y. Ihara, M. Munekata, K. Ishige, T. Noguchi, *J. Biosci. Bioeng.* **2001**, *91*, 557–563.

[6] G. F. Bickerstaff, *Immobilization of Enzymes and Cells*, Humana Press, Totowa, **1997**, p. 367.

[7] a) C. Augé, S. David, C. Mathieu, C. Gautheron, *Tetrahedron Lett.* **1984**, *25*, 1467–1470; b) C. Augé, S. David, C. Gautheron, *Tetrahedron Lett.* **1984**, *25*, 4663–4664.

[8] C. Augé, A. Malleron, H. Tahrat, A. Marc, J. L. Goergen, M. Cerutti, W. F. A. Steelant, P. Delannoy, A. Lubineau, *Chem. Commun.* **2000**, 2017–2018.

[9] a) X. Chen, J. Fang, J. Zhang, Z. Liu, P. Andreana, P. Kowal, P. G. Wang, *J. Am. Chem. Soc.* **2001**, *123*, 2081–2082; b) Z. Liu, J. Zhang, X. Chen, P. G. Wang, *ChemBioChem* **2002**, *3*, 348–355.

[10] a) T. Bulter, L. Elling, *Glycoconjugate J.* **1999**, *16*, 147–159; b) Y. Ichikawa, R. Wang, C. H. Wong, *Methods Enzymol.* **1994**, *247*, 107–127.

[11] a) T. Boller, Ch. Meier, S. Menzler, *Org. Process Res. Dev.* **2002**, *6*, 509–519; b) C. Mateo, G. Fernandez-Lorente, O. Abian, R. Fernandez-Lafuente, J. M. Guisan, *Biomacromolecules* **2000**, *1*, 739–745.

[12] C. Mateo, O. Abian, R. Fernandez-Lafuente, J. M. Guisan, *Biotechnol. Bioeng.* **2000**, *68*, 98–105.

[13] G. Czichocki, H. Dautzenberg, E. Capan, K. D. Vorlop, *Biotechnol. Lett.* **2001**, *23*, 1303–1307.

[14] T. Coradin, E. Mercey, L. Lisnard, J. Livage, *Chem. Commun.* **2001**, 2496–2497.

[15] a) F. H. Arnold, *Bio/Technology* **1991**, *9*, 151–156; b) R. R. Beitle, M. M. Ataai, *Biotechnol. Prog.* **1993**, *9*, 64–69.

[16] P. Tomme, N. R. Gilkes, M. M. Guarna, C. A. Haynes, D. Hasenwinkle, E. Jervis, P. Johnson, L. Mcintosh, R. A. J. Warren, D. G., *Ann. N.Y. Acad. Sci.* **1996**, *799*, 418–424.

[17] a) O. Shoseyov, M. Takagi, M. A. Goldstein, R. H. Doi, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3483–7; b) O. Shoseyov, R. A. J. Warren, *inNovations* **1997**, *7*, 1–3.

[18] M. A. Goldstein, M. Takagi, S. Hashida, O. Shoseyov, R. O. Doi, I. H. Segel, *J. Bacteriol.* **1993**, *175*, 5762–5768.

[19] Z. Fujimoto, A. Kuno, S. Kaneko, H. Kobayashi, I. Kusakabe, H. Mizuno, *J. Mol. Biol.* **2002**, *316*, 65–78.

[20] B. K. Tibbot, D. W. S. Wong, G. H. Robertson, *J. Protein Chem.* **2000**, *19*, 663–669.

[21] K. Momma, B. Mikami, Y. Mishima, W. Hashimoto, K. Murata, *J. Mol. Biol.* **2002**, *316*, 1051–1059.

[22] a) M. Farooqi, M. Saleemuddin, R. Ulber, P. Sosnitza, T. Scheper, *J. Biotechnol.***1997**, *55*, 171–179; b) D. Mislovicova, P. Gemeiner, J. Sandula, J. Masarova, A. Vikartovska, P. Docolomansky, *Biotechnol. Appl. Biochem.* **2000**, *31*, 153–159.

[23] X. Chen, P. R. Andreana, P. G. Wang, *Curr. Opin. Chem. Biol.* **1999**, *3*, 650–658.

[24] a) M. G. Peter, C. A. Lingwood, *Biochim. Biophys. Acta* **2000**, *1501*, 116–124; b) F. D. Barnett, M. Abul-Milh, M. Huesca, C. A. Lingwood, *Infect. Immun.* **2000**, *68*, 3108–3115; c) C. A. Lingwood, *Biosci. Rep.* **1999**, *19*, 345–354; d) C. A. Lingwood, *Biochim. Biophys. Acta* **1999**, *1455*, 375–386.

[25] R. R. Beitle, M. M. Ataai in *Immobilization of Enzymes and Cells* (Ed.: G. F. Bickerstaff), Humana Press, Totowa, **1997**, pp. 339–343.

[26] X. Chen, Z. Liu, J. Zhang, W. Zhang, P. Kowal, P. G. Wang, *ChemBioChem* **2002**, *3*, 47–53.

[27] a) S. Koizumi, T. Endo, K. Tabata, A. Ozaki, *Nature Biotechnol.* **1998**, *16*, 847–348; b) T. Endo, S. Koizumi, K. Tabata, S. Kakita, A. Ozaki, *Carbohydr. Res.* **1999**, *316*, 179–183; c) T. Endo, S. Koizumi, K. Tabata, S. Kakita, A. Ozaki, *Carbohydr. Res.* **2001**, *330*, 439–443; d) T. Endo, S. Koizumi, K. Tabata, A. Ozaki, *Appl. Microbiol. Biotechnol.* **2000**, *53*, 257–261.

[28] a) U. Prusse, B. Fox, M. Kirchhoff, F. Bruske, J. Breford, K. D. Vorlop, *Biotechnol. Tech.* **1998**, *12*, 105–108; b) H. Brandenberger, F. Widmer, *J. Biotechnol.* **1998**, *63*, 73–80.

[29] a) P. Gemeiner, Ľ. Kurillová, O. Markovič, A. Malovíková, D. Uhrín, M. Ilavský, V. Štefuca, M. Polakovič, V. Báleš, *Biotechnol. Appl. Biochem.* **1991**, *13*, 335–345; b) M. Navrátil, P. Gemeiner, J. Klein, E. Šturdík, A. Malovíková, J. Nahálka, Z. Dömény, D. Šmogrovičová, *Artif. Cells Blood Substitutes Immobilization Biotechnol.* **2002**, *30*, 119–218.

[30] a) V. L. Lozinsky, F. M. Plieva, *Enzyme Microb. Technol.* **1998**, *23*, 227–242; b) U. Jahnz, P. Wittlich, U. Prüsse, K. D. Vorlop in *Focus on Biotechnology*, *Vol. 4* (Eds.: M. Hofman, P. Thonart), Kluwer Academic Publishers, Dordrecht, **2001**, pp. 293–307; c) M. Jekel, A. Buhr, K. D. Vorlop, *Chem. Eng. Technol.* **1998**, *21*, 275–278.

[31] a) J. A. Francisco, C. Stathopoulos, R. A. Warren, D. G. Kilburn, G. Georgiou, *Bio/Technology* **1993**, *11*, 491–495; b) A. A. Wang, A. Mulchandani, W. Chen, *Biotechnol. Prog.* **2001**, *17*, 407–411; c) J. Lehtiö, H. Wernerus, P. Samuelson, T. T. Teeri, S. Stahl, *FEMS Microb. Lett.* **2001**, *195*, 197–204.

  0947-6539/03/0902-0377 $ 20.00+.50/0