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Recent progress in chemoenzymatic synthesis of human glycans

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Glycan is an essential cell component that usually exists in either a free form or a glycoconjugated form. Glycosylation affects the regulatory function of glycoconjugates in health and disease development, indicating the key role of glycan in organisms. Because of the complexity and diversity of glycan structures, it is challenging to prepare structurally well-defined glycans, which hinders the investigation of biological functions at the molecular level. Chemoenzymatic synthesis is an attractive approach for preparing complex glycans, because it avoids tedious protecting group manipulations in chemical synthesis and ensures high regio- and stereo-selectivity of glucosides during glycan assembly. Herein, enzymes, such as glycosyltransferases (GTs) and glycosidases (GHs), and sugar donors involved in the chemoenzymatic synthesis of human glycans are initially discussed. Many state-of-the-art chemoenzymatic methodologies are subsequently displayed and summarized to illustrate the development of synthetic human glycans, for example, *N*- and *O*-linked glycans, human milk oligosaccharides, and glycosaminoglycans. Thus, we provide an overview of recent chemoenzymatic synthetic designs and applications for synthesizing complex human glycans, along with insights into the limitations and perspectives of the current methods.

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Introduction

Glycan is one of the most complicated and diverse molecules found in organisms, and it is free or covalently linked to other molecules to form glycoconjugates, such as glycoproteins, proteoglycans, glycolipids, and glycoRNAs.[1,2] Glycosylation regulates various biological processes, including signal transduction, cell migration, inflammation, immune evasion, cancer metastasis, and bacterial and viral infection.[3–6] However, the mechanism of action used by glycans in many biological events remains unclear. One substantial reason for this is the lack of structurally well-defined glycans. The complexity and diversity of glycan structures considerably hinder the synthesis of structurally well-defined glycans. In contrast to the synthesis of nucleic acids and proteins, the biosynthesis of glycans is a non-template-driven process. The expression levels of glycosyltransferases (GTs) and glycosidases (GHs)[7] and the availability of sugar nucleotides can affect the formation of glycans *in vivo*.[8–10]

Extraction from natural sources[11] and artificial synthesis *in vitro* are the two main approaches for obtaining glycans. Because of the low abundance and micro-heterogeneity of endogenous glycans, it is difficult to obtain structurally well-defined glycans from nature for application in biological studies, despite using state-of-the-art analytical tools. In contrast, artificial synthesis is an attractive alternative method for preparing glycans. Chemical synthesis can be used to produce various natural or non-natural glycans and glycan derivatives.[12–14] However, it is difficult and time-consuming to create scale-prepared complex glycans because of the tedious protection of group manipulations and challenging stereoselective glycosylation that must be performed.

Enzymatic glycosylation is another synthetic method used to produce glycans.[15,16] One of its advantages is its ability to produce highly regio- and stereo-selective glycosidic bonds without protecting group participation. However, one enzyme, for example one GT, can usually catalyze the formation of one glycosidic bond. Thus, the availability of enzymes limits the application of this method. To address these limitations, a chemoenzymatic synthesis strategy combining chemical synthesis and enzymatic glycosylation was developed and has been widely used in the past few decades to synthesize complex glycans.[17,18] There is no need for tedious protecting group manipulations in chemical synthesis with this novel synthetic method, and it ensures high regio- and stereo-selectivity of glucosides during glycan assembly. As a result, it offers great potential for the efficient synthesis of highly complex branched glycans (Fig. 1). This mini-review provides an overview of the typical chemoenzymatic synthetic methods used to produce complex human glycans in recent years.

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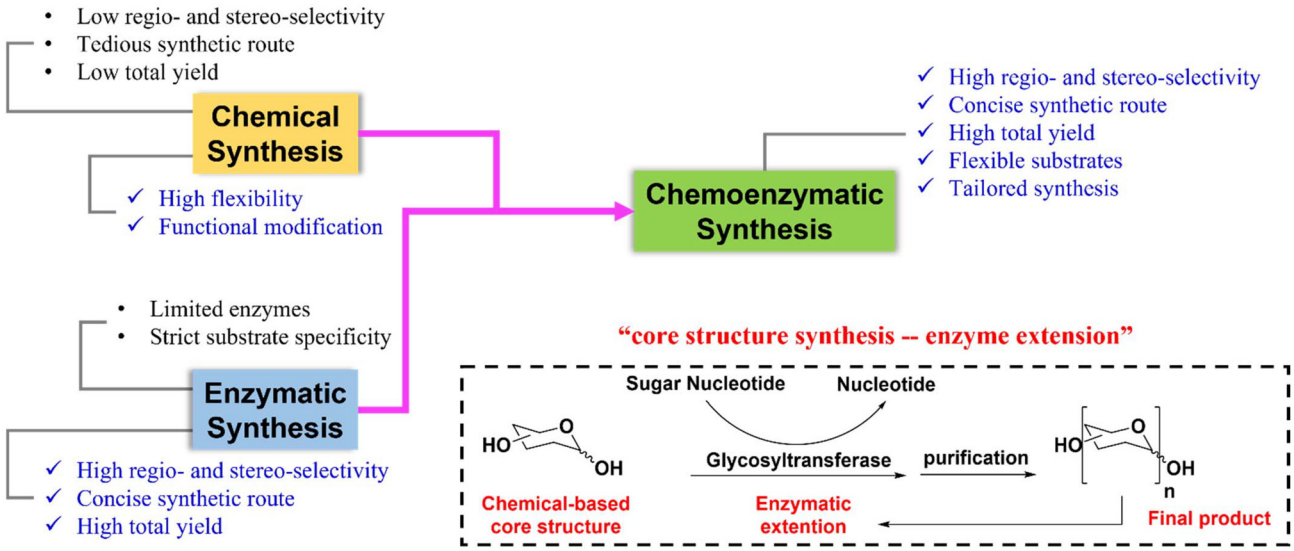

Fig. 1 Comparison between chemical or enzymatic strategies and chemoenzymatic strategy, and a schematic illustration of the 'Core structure synthesis – enzyme extension' synthetic strategy.

Enzymes and sugar donors

GTs and GHs are two major enzymes applied to the (chemo) enzymatic synthesis of glycans. GTs use nucleotide sugars as activated donors to natively catalyze the biosynthesis of glycans and glycoconjugates,[19] and are the most widely used in glycan synthesis. The Carbohydrate-Active Enzymes (CAZy) database (**https://www.cazy.org**) groups more than 1 000 000 GTs into 135 families (as of 30th May 2024), with an additional 60 000 GT sequences remaining unclassified. Although hundreds of thousands of GTs have been discovered, the members successfully used in the chemoenzymatic synthesis of human glycans are mainly glucosyltransferases (GlcTs), *N*-acetylglucosaminyltransferases (GlcNAcTs), glucouronyltransferases (GlcATs), galactosyltransferases (GalTs), *N*-acetylgalactosaminyltransferases (GalNAcTs), mannosyltransferases (ManTs), sialyltransferases (SiaTs), and fucosyltransferases (FucTs).

GTs used in chemoenzymatic synthesis should have the following ideal properties: high expression level, high stability during storage and application, high activity, high regio- and stereo-selectivity, minimal undesirable activities, and promiscuity towards modified substrate analogs.[20] However, it is unrealistic to expect that all the ideal properties will exist for wild-type GTs. To address this, prokaryotic and eukaryotic expression systems[21,22] have been constructed and combined with the enzyme engineering technique to generate GTs and GT mutants better suited for synthesis.

GHs, also called glycoside hydrolases, are enzymes that hydrolyze the glycosidic linkages in glycans and glycoconjugates.[23] GHs are usually classified into *exo*-glycosidases and *endo*-glycosidases based on their cleavage sites. Exo-glycosidases individually hydrolyze nonreducing saccharide units, whereas *endo*-glycosidases hydrolyze internal glycosidic bonds to cleave more than one sugar residue.[24] The hydrolysis feature of GHs has been widely utilized as a complementary tool in the chemoenzymatic synthesis of glycans. For example, a well-designed *N*-glycan precursor was treated with different GHs and chemical deprotection to provide a high mannose-type *N*-glycan library.[25] Most importantly, the hydrolytic activity of GHs is reversible, which implies that GHs possess the potential to synthesize glycosides.

Many oligosaccharides and glucosides have been successfully synthesized utilizing GHs that intrinsically are able to perform transglycosylation capability.[26–28] In addition, many mutational GHs presented many more highly efficient transglycosylations in the synthesis of glycans,[29,30] glycoproteins,[31] and the glycoengineering of antibodies.[32] Mutational GHs have also been used for cell surface transglycosylation for the site-specific analysis of glycosylation of glycoproteins.[33–35] Furthermore, some GHs can be engineered into mutants, called glycosynthases, which are used in glycan synthesis and prevent glycosidic bond hydrolysis.[36] Many excellent reports provide a superb overview of the application of GHs and glycosynthases in biocatalytic glycoside synthesis.[37–39] The current review focuses on the development of the biomimetic synthesis of human glycans using activated donors and GTs.

Monosaccharides need to be converted to the activated form (sugar donors) before being recognized and transferred by GTs to build glycans.[40] Sugar donors usually include sugar nucleotides, dolichol phosphate sugars, and specific monosaccharide analogs. Sugar nucleotides are the most commonly used sugar donors in the biosynthesis and chemoenzymatic synthesis of glycans. Most GTs recognize sugar nucleotides as the donor for glycosylation,[41] while a few GTs recognize dolichol phosphate sugar, which is present in the initial stages of

glycan biosynthesis in the endoplasmic reticulum.[42] In addition, phosphorylated sugars and fluoride sugars are often recognized by glycosynthases.[43,44]

In humans, there are nine common sugar nucleotides, namely, UDP-Glc, UDP-GlcNAc, UDP-GlcA, UDP-Gal, UDP-GalNAc, UDP-Xyl, GDP-Man, GDP-Fuc, and CMP-Neu5Ac (Fig. 2a). The formation of sugar nucleotides *in vivo* is complicated and involves multiple pathways with multi-enzymes.[45,46] Therefore, the biomimetic synthesis of sugar nucleotides *in vitro* is challenging. To achieve the large-scale preparation of sugar nucleotides, one-pot multi-enzyme strategies[47,48] and *in situ* sugar nucleotide regeneration systems[49,50] have been developed. Wen and coworkers reported an efficient cofactor-driven cascade conversion strategy for preparing several rare

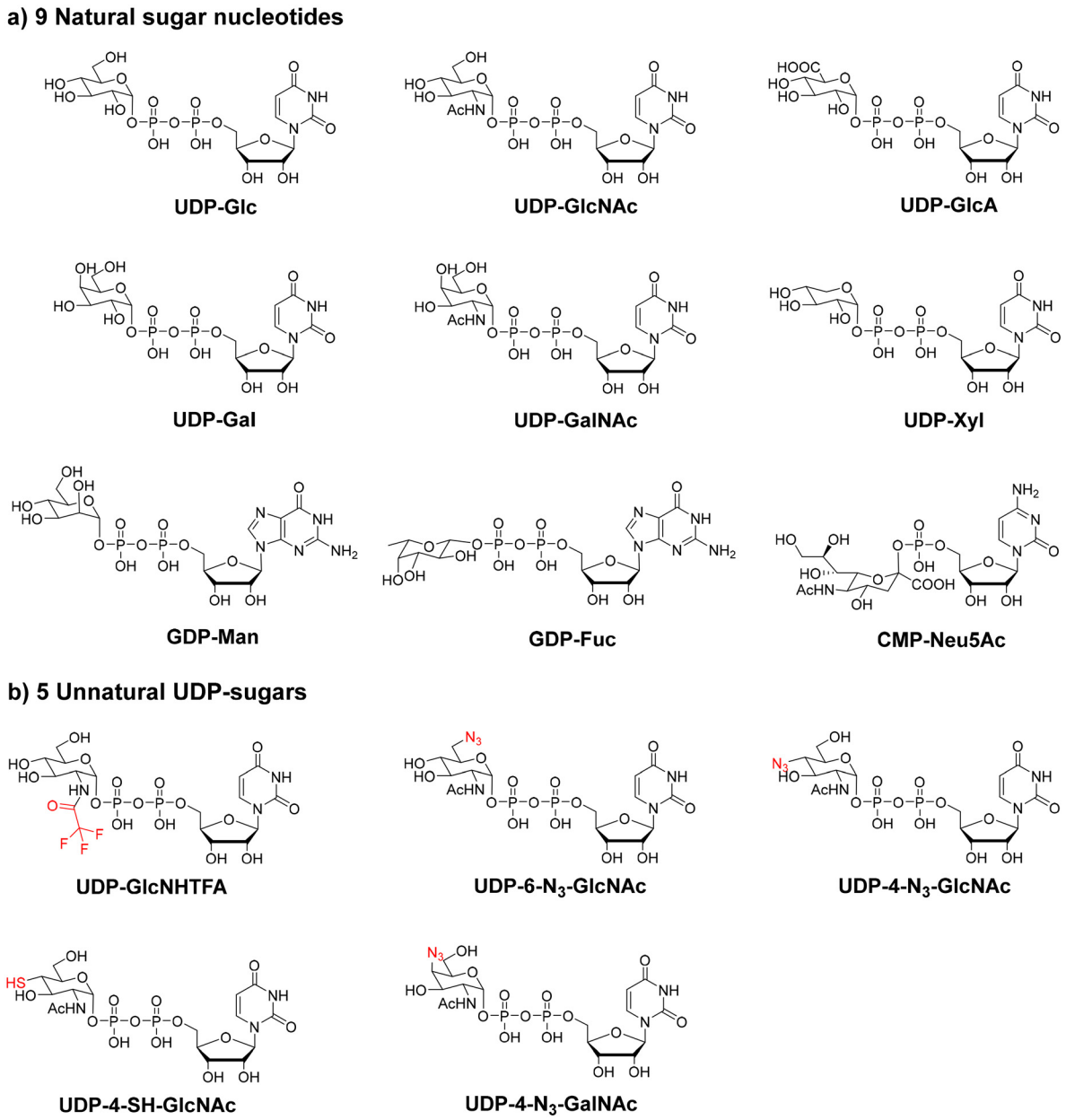

Fig. 2 Nine common sugar nucleotides in humans and five unnatural UDP-sugar analogs. (a) Uridine-5′-diphosphate-glucose (UDP-Glc); uridine-5′-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc); uridine-5′-diphosphate-glucuronic acid (UDP-GlcA); uridine-5′-diphosphate-galactose (UDP-Gal); uridine-5′-diphosphate-*N*-acetylgalactosamine (UDP-GalNAc); uridine-5′-diphosphate-xylose (UDP-xyl); guanosine-5′-diphosphate-mannose (GDP-Man); guanosine-5′-diphosphate-fucose (GDP-Fuc); and cytidine-5′-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac). (b) Uridine 5′-diphosphate-*N*-trifluoroacetylglucosamine (UDP-GlcNHTFA); uridine-5′-diphosphate-6-azid-*N*-acetylglucosamine (UDP-6-N_3-GlcNAc); uridine-5′-diphosphate-4-azid-*N*-acetylglucosamine (UDP-4-N_3-GlcNAc); uridine-5′-diphosphate-4-thio-*N*-acetylglucosamine (UDP-4-SH-GlcNAc), and uridine-5′-diphosphate-4-azid-*N*-acetylgalactosamine (UDP-4-N_3-GalNAc).

sugar nucleotides[10,51,52] that are important synthetic precursors for many rare sugar-containing glycans.

Unnatural sugar nucleotides (structural analogs of sugar nucleotides) have been designed and prepared for glycan synthesis purposes. They can be recognized by GTs, which then incorporate unnatural sugar residues into glycan chains. Glycans containing unnatural sugar residues can be further manipulated to achieve precise structural control. For example, uridine 5′-diphosphate-*N*-trifluoroacetylglucosamine (UDP-GlcNHTFA) has been widely used as an analog of UDP-GlcNAc in glycan synthesis.[53–55] In addition, UDP-6-N_3-GlcNAc, UDP-4-N_3-GlcNAc, UDP-4-SH-GlcNAc, and UDP-4-N_3-GalNAc were also successfully prepared and used in the chemoenzymatic synthesis of glycans (Fig. 2b).[48,56,57] Another application of unnatural sugar nucleotides is *in situ* synthesis of cell surface glycans to achieve glycan visualization and labeling.[58–60]

N-Linked glycans

Human *N*-glycans can be structurally divided into complex *N*-glycan, hybrid *N*-glycan, and high-mannose *N*-glycan, which have a common core pentasaccharide structure ($Man_3GlcNAc_2$) attached to the typical Asn-X-Ser/Thr sequon of protein with an *N*-glycosidic bond.[5,61–64] Obtaining the core pentasaccharide is a prerequisite for the chemoenzymatic synthesis of *N*-glycans. The current chemoenzymatic methods involve the initial preparation of the core structure, and then its enzymatic extension.[65–68] Following the concept of 'core structure synthesis-enzyme extension', numerous efficient chemoenzymatic synthetic methods have been developed for the synthesis of symmetric and asymmetric *N*-glycans.

Wong and co-workers were among the first to elongate *N*-glycans using GTs. They later designed and developed a synthetic strategy called the modular assembly method.[69,71,72] In this method, acceptor and donor modules are chemically synthesized. Thereafter, the coupling of both modules installs the core structure of *N*-glycans. Finally, enzymatic extension and modification are performed to achieve the production of targeted *N*-glycans (Fig. 3a). Although chemical manipulations are challenging, this method allows for the synthesis of many complex *N*-glycans. *N*-Glycans are usually asymmetrically substituted with a unique saccharide appendage at each branching point.[5,73]

Many efforts have been made to synthesize asymmetrical *N*-glycans, resulting in their easy access for experimentation.[65,74,75] In 2013, Boons and co-workers reported a general strategy for the chemoenzymatic synthesis of asymmetrically branched *N*-glycans.[65] In this work, a core pentasaccharide modified by orthogonal protecting groups, including levulinoyl (Lev), fluorenylmethyloxycarbonate (Fmoc), allyloxycarbonate (Alloc), and 2-naphthylmethyl (Nap) groups, at potential branching positions was synthesized as a common synthetic precursor. This allowed for the selective attachment of specific saccharide moieties by chemical glycosylation, followed by branch elongation by GTs to produce libraries of asymmetrical *N*-glycans (Fig. 3b). In the same year, Ito *et al.* reported a top-down chemoenzymatic strategy to produce a library of high mannose-type *N*-glycans.[25] Similarly, other methods have been reported to synthesize various *N*-glycans.[76–79]

Trimming sialoglycopeptide (SGP) extracted from egg or egg yolk powder by GHs is another method used to obtain the core structure of *N*-glycans.[80–82] Boons and co-workers developed a "stop and go" strategy, starting from SGP, to synthesize asymmetrical multi-antennary *N*-glycans.[55] This method allows for the conversion of a bi-antennary heptasaccharide into multi-antennary *N*-glycans within ten or fewer chemical and enzymatic steps. In this work, UDP-GlcNHTFA was employed as an analog of UDP-GlcNAc to attach GlcNHTFA to the bi-antennary heptasaccharide from SGP through the catalysis of human mannosyl-glycoprotein *N*-acetylglucosaminyltransferases (MGAT4 and MGAT5), forming additional controllable arms on the core structure of *N*-glycan.

The GlcNHTFA can be converted to glucosamine ($GlcNH_2$) under moderate basic conditions. $GlcNH_2$ can further be converted to 2-deoxy-2-azido-glucose ($GlcN_3$). The $GlcNH_2$ and $GlcN_3$ arms are not recognized by human β-1,4-galactosyltransferase (B4GalT1), thus allowing regio-selective galactosylation of GlcNAc. Finally, the $GlcNH_2$ and $GlcN_3$ residues can be easily converted back to GlcNAc, which is a suitable galactosylation site for B4GalT1 (Fig. 3c). Another study proved that the human *N*-acetylglucosaminyltransferases MGAT1 and MGAT2 can also use UDP-GlcNHTF as a donor. Thus, a new "stop and go" chemoenzymatic methodology was developed to prepare 32 asymmetrical bi-antennary *N*-glycans found in airway tissues.[83]

The large-scale production of core pentasaccharide, a key component for synthesizing various *N*-glycans, has been challenging due to difficulties in obtaining it through chemical synthesis or isolation from nature. Although many excellent studies have identified the catalytic roles of human mannosyltransferases Alg1 and Alg2, which are key enzymes in the biosynthesis of the core pentasaccharide, large-scale preparation of the core structure has been unsuccessful.[84–86]

To address this issue, Wen and coworkers recently developed a general platform for the concise chemoenzymatic synthesis of *N*-glycans.[70] In this study, an inexpensive commercially available disaccharide GlcNAcβ1,4GlcNAc was chemically treated and coupled with an 11-carbon lipid tail (undecanol) to produce the common starting materials. This starter is well recognized as an acceptor by recombinant Alg1. Furthermore, the mannosylated starter is also recognized by recombinant Alg2, which is responsible for the installation of α-1,3- and α-1,6-mannose into the core pentasaccharide. Based on these, large-scale core pentasaccharides were efficiently synthesized using a few facile chemical and enzymatic steps. Starting from the synthetic core structure, more than 60 *N*-glycans were successfully synthesized (Fig. 3d).

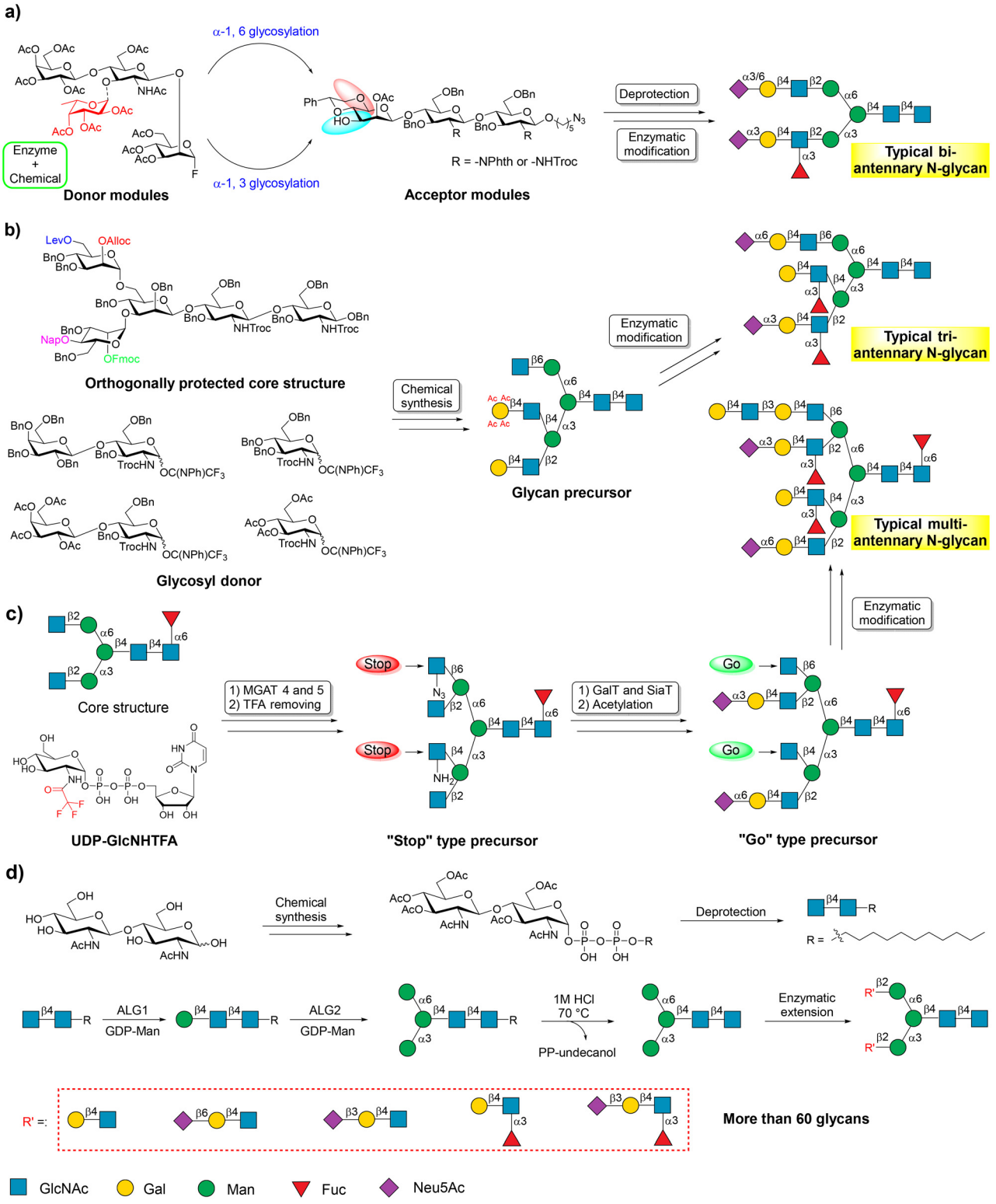

Fig. 3 Chemoenzymatic strategies for the synthesis of *N*-glycans. (a) Modular assembly strategy for the synthesis of *N*-glycans. Reproduced with permission from Shivatare *et al.*[69] Copyright 2016, Nature Publishing Group. (b) Orthogonally protected synthetic strategy along with enzymatic modification for the synthesis of asymmetrically branched *N*-glycans. Reproduced with permission from Wang *et al.*[65] Copyright 2013, American Association for the Advancement of Science. (c) The "stop and go" strategy for chemoenzymatic synthesis of complex *N*-glycans. Reproduced with permission from Liu *et al.*[55] Copyright 2018, Nature Publishing Group. (d) Large-scale core structure-based concise chemoenzymatic synthesis of *N*-glycans.[70] Copyright 2024, Elsevier Inc. The representation symbol of glucosamine should be " " according to the guidance of Symbol Nomenclature for Glycans (SNFG). We use " " in place of " " in (c) to clearly show the conversion of functional groups.

In addition, many biologically related antibodies equipped with *N*-glycans have proven that *N*-glycosylation on antibodies exerts a significant role in their effector function.[87–89] Therefore, chemoenzymatic methods have also been exploited to prepare a panel of *N*-glycans found on human serum immunoglobulin G (IgG). In 2020, Unverzagt and co-workers successfully galactosylated the bisecting GlcNAc of rare *N*-glycans identified in IgG.[90] In this study, four multi-antennary *N*-glycans were treated with a bovine β-1,4-galactosyltransferase. Only bi-antennary bisected *N*-glycans can be galactosylated. Li and co-workers constructed a 64-membered IgG *N*-glycan library starting from SGP.[91] The *N*-glycan remodeling of antibodies using enzymes, such as *endo*-β-*N*-acetylglucosaminidase mutants, for producing engineered antibodies with homogeneous *N*-glycan forms has been reviewed in recent years and is not discussed here.[31,92,93]

O-Linked glycans

O-GalNAc and *O*-mannose are two major *O*-linked glycan forms in humans.[94,95] They are the focus of many chemoenzymatic strategies.[96,97] A regioselective one-pot multienzyme (OPME) chemoenzymatic strategy was developed to systematically synthesize the sialyl Core 2 glycans.[98] This study exploited the restricted acceptor specificity of sialyltransferases (*Pasteurella multocida* α-2,3-sialyltransferase 3) and the rational design of acceptors to successfully achieve the synthesis of regioselective sialylated Core 2 glycans (Fig. 4a).

In addition to the regioselective challenge, site-specific modification, such as sulfation of *O*-GalNAc glycans, complicates the synthesis process. Li and coworkers chemically synthesized sulfated and non-sulfated Core 2 trisaccharides as starters to enzymatically prepare over 30 structurally well-defined Core 2 glycans (Fig. 4b).[99] In addition, other core structures of *O*-GalNAc were synthesized using a robust chemoenzymatic modular assembly strategy.[100] This method focuses on exploiting three chemical building blocks to convergently assemble the *O*-GalNAc Core 1–4 and Core 6 structures.

Enzymatic decoration was then applied to create a series of *O*-GalNAc glycans. A total of 83 *O*-GalNAc glycans covering different natural glycan epitopes was synthesized in this study, and a glycan microarray was constructed to identify various lectins, anti-glycan antibodies, and serum samples from patients with colorectal cancer. Recently, three α-linked rare cores (Core 5, Core 7, and Core 8) and their sialylated forms were prepared by Li's group.[103] The *Photobacterium damselae* α-2,6-sialyltransferase (Pd2,6ST) demonstrated restricted substrate specificity toward *O*-GalNAc glycan cores 5, 7, and 8, selectively installing α-2,6 sialic acid in the 6-OH of the internal GalNAc residue of Cores 5 and 8, and in the 6-OH of the terminal GalNAc residue of Core 7. These methods greatly facilitated the preparation of many structurally diverse *O*-GalNAc glycans, which further deepened the biological function study of *O*-GalNAc glycans.

The chemoenzymatic synthesis of *O*-mannosyl glycans is also performed using rational methodology designing. The *O*-mannosyl glycans are usually bi-antennary structures, in which two GlcNAc residues bearing further elaborations are each linked to core mannose with β-1,2- and β-1,6-glycosidic bonds.[95] Achieving the regioselective chemoenzymatic synthesis of *O*-mannosyl glycans is challenging. Cao and co-workers successfully achieved the diversity-oriented assembly of 58 complex *O*-mannosyl glycans starting from five chemically synthesized core structures.[101] Five synthesized core structures determined the regioselective sialylations and fucosylations of all of the complex glycans. Among these diverse glycans, 55 glycans were first synthesized (Fig. 4c).

Li and coworkers developed a scaffold synthesis/enzymatic extension strategy to efficiently synthesize 45 *O*-mannosyl glycans derived from human beings.[104] Because muscular dystrophy-related α-dystroglycan (α-DG) is often mannosylated,[105] *O*-mannosyl α-DG is an attractive biomarker for disease in clinical settings.[106] Peng and coworkers prepared several *O*-mannosylated glycopeptides using a microwave-assisted SPPS method.[107] The human natural killer-1 (HNK-1) epitope, 3-*S*-GlcAβ1,3Galβ1,4GlcNAc trisaccharide, is a unique component of *O*-mannosyl glycans in brain tissues. Cao and co-workers first synthesized a series of HNK-1-bearing *O*-mannosyl glycans using a judicious chemoenzymatic synthesis approach.[102] In this method, a chemically synthesized trisaccharide lactone donor was converted into three HNK-1-containing branched *O*-mannosyl precursors *via* chemical glycosylation, selective cleavage of lactone, and sulfation. These precursors were enzymatically elaborated, taking advantage of substrate promiscuities of bacterial GTs to produce various complex HNK-1-bearing *O*-mannosyl glycans (Fig. 4d).

Poly-LacNAc glycans

Poly-*N*-acetyl lactosamine (poly LacNAc) glycans consist of type II LacNAc repeating units and are common structural motifs of various complex human glycans, such as *N*- and *O*-linked glycans, and human milk oligosaccharides (HMOs). The fucosylation and sialylation of poly LacNAc glycans produce biologically related glycan epitopes, such as Lewis X or sialyl Lewis X. The synthesis of regioselective glycosylated poly LacNAc glycans is an attractive topic. Taking advantage of sequential enzymatic and chemical reactions, galactose oxidase, and reductive amination, Elling and coworkers chemoenzymatically synthesized a panel of branched LacNAc oligomers bearing LacNAc and/or *N*′,*N*″-diacetyllactosamine (LacdiNAc) glycan epitopes (Fig. 5a).[108] Oxidation by galactose oxidase converted LacNAc to 6′-aldehyde LacNAc, which can be recognized by *Helicobacter pylori* β-3-*N*-acetylglucosaminyltransferase (β3GlcNAcT) as the acceptor to elongate the LacNAc chain. The reserved aldehyde site was used for coupling with amine-linked glycans *via* reductive amination.

In 2019, Cao's group described a reprogrammed enzymatic assembly line for regioselective fucosylation of the poly

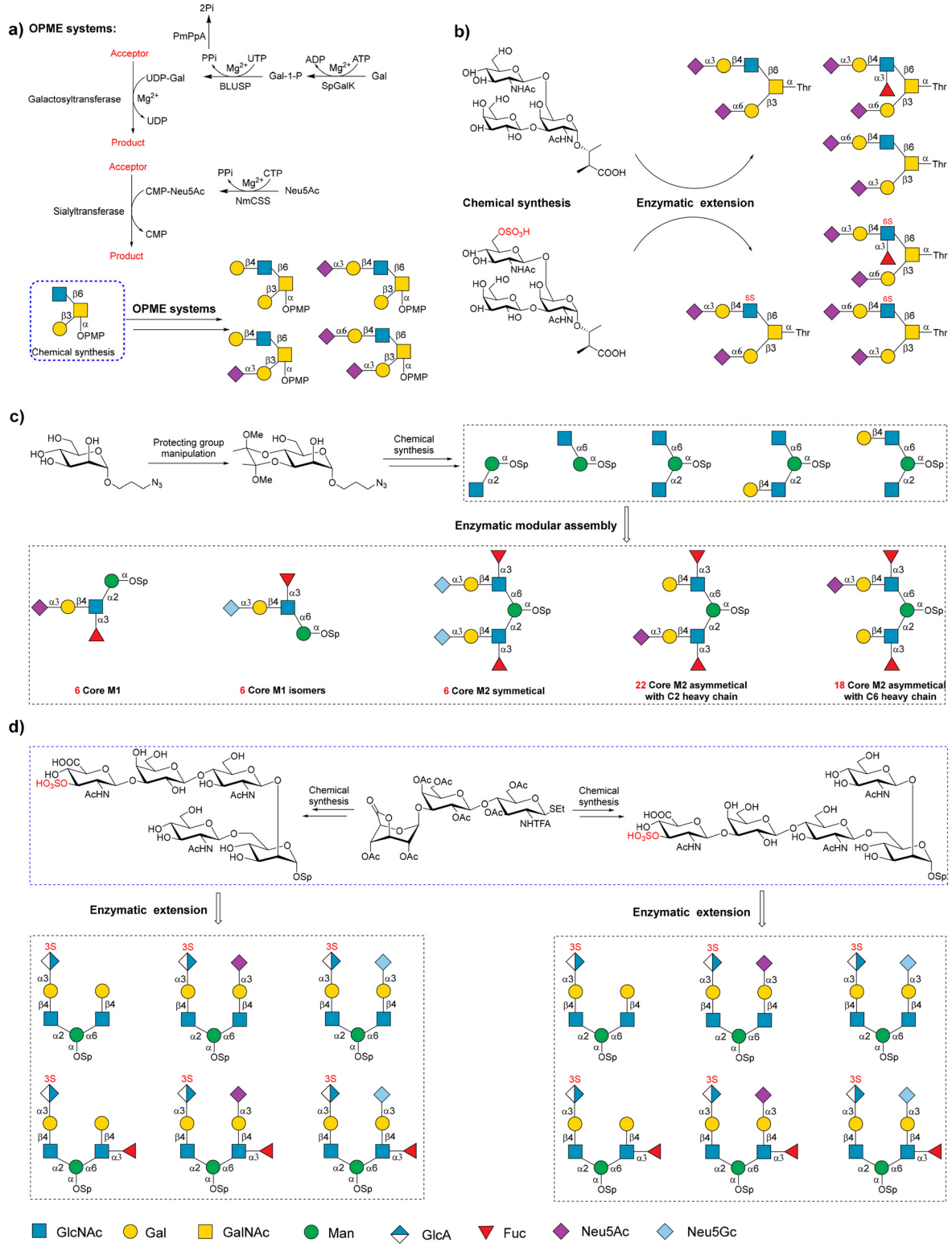

Fig. 4 Chemoenzymatic strategies for the synthesis of *O*-GalNAc and *O*-mannose glycans. (a) Regioselective one-pot multienzyme (OPME) chemoenzymatic strategies for the synthesis of sialyl Core 2 glycans. SpGalK: *Streptococcus pneumoniae* TIGR4 galactokinase. BLUSP: UDP-sugar pyrophosphorylase from *Bifidobacterium longum*. PmPPA: pyrophosphatase from *Pasteurella multocida*. NmCSS: *Neisseria meningitidis* CMP-sialic acid synthetase. Reproduced with permission from Santra *et al.*[98] Copyright 2018 American Chemical Society. (b) Diversity-oriented chemoenzymatic approach for the preparation of sulfated/nonsulfated Core 2 glycans. Reproduced with permission from Xu *et al.*[99] Copyright 2021 American Chemical Society. (c) Chemoenzymatic assembly of mammalian *O*-mannosyl glycans. Reproduced with permission from Meng *et al.*[101] Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (d) Chemoenzymatic synthesis of a sulfated HNK-1 epitope bearing *O*-mannosyl glycans. Reproduced with permission from Gao *et al.*[102] Copyright 2019 American Chemical Society.

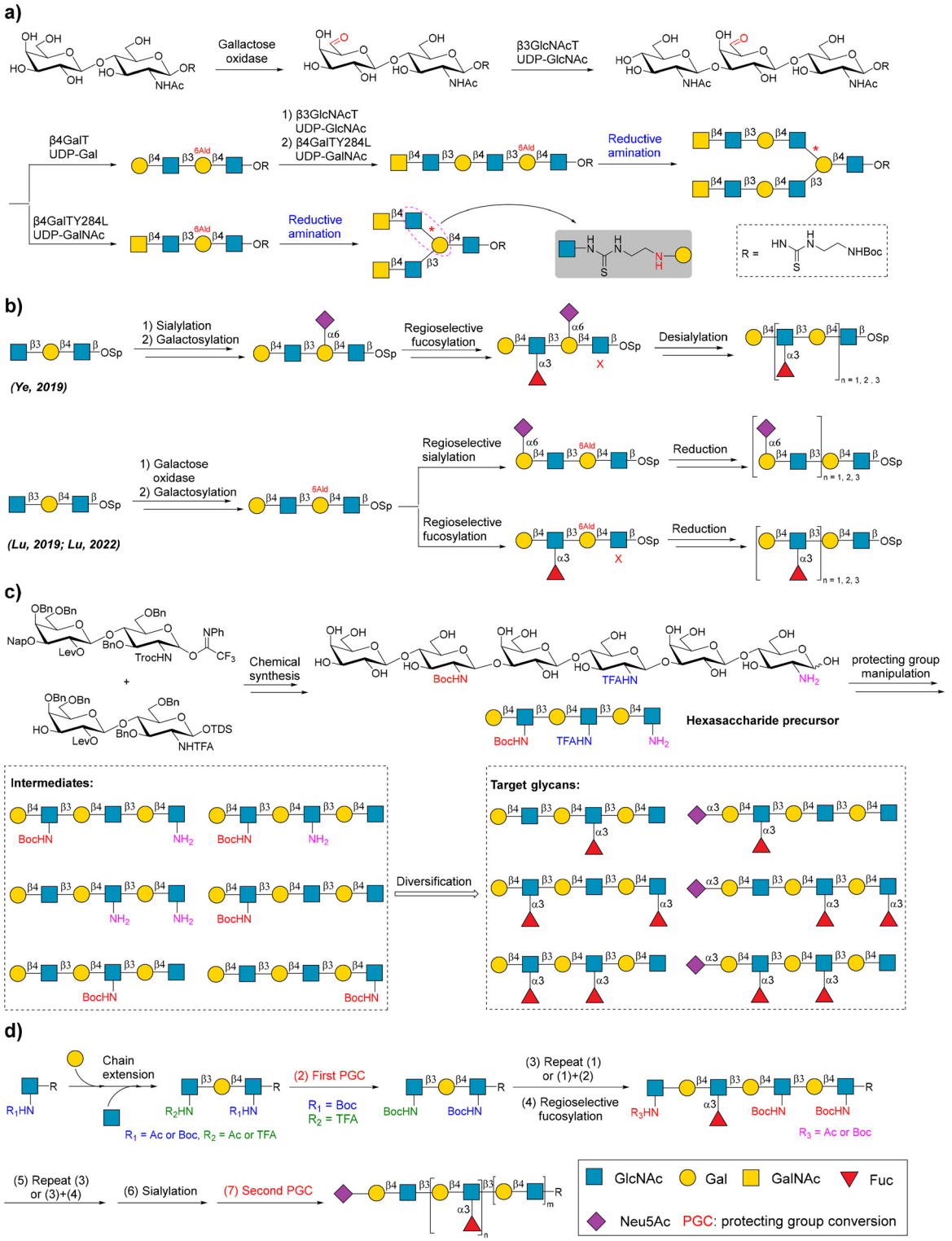

Fig. 5 Chemoenzymatic strategies for the synthesis of poly LacNAc glycans. (a) Chemoenzymatic synthesis of branched poly LacNAc glycans. Reproduced with permission from Laaf *et al.*[108] Copyright 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Glycosylation and GOase-controlled regioselective synthesis of poly LacNAc glycans. Reproduced with permission from Ye *et al.*, Lu *et al.*, and Lu *et al.*[109–111] Copyright 2019, Nature Publishing Group. Copyright 2019 American Chemical Society. Copyright 2022 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (c) Unnatural substrate-controlled regioselective synthesis of poly LacNAc glycans. Reproduced with permission from Gagarinov *et al.*[112] Copyright 2022 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (d) Acceptor-mediated regioselective fucosylation of poly LacNAc glycans. Reproduced with permission from Tseng *et al.*[114] Copyright 2023 American Chemical Society. The representation symbol of glucosamine should be " " according to the guidance of Symbol Nomenclature for Glycans (SNFG). We use " " in place of " " in (c) to clearly show the conversion of functional groups.

LacNAc backbone.[109] In this study, the α-2,6-linked sialic acid on LacNAc repeating units was used as the protecting group against fucosylation of LacNAc repeating units. Thus, the fucosylation of poly-LacNAc glycans was precisely controlled in a site-specific manner. Finally, the unwanted sialic acid can be removed by the use of sialidase. In the same year, they also reported a general redox-controlled site-specific sialylation strategy.[110] This strategy used galactose oxidase to selectively oxidize the C-6-hydroxyl group of Gal, solving the problem of regioselective sialylation of poly LacNAc glycans. Recently, this redox-controlled glycosylation strategy was used for the site-specific enzymatic fucosylation of many *N*- and *O*-linked glycans (Fig. 5b).[111]

The exploitation of protecting group manipulation-based glycosyl donors also achieved regioselective sialylation and fucosylation of poly LacNAc glycans. Boons and coworkers developed a regioselective chemoenzymatic strategy that produced various fucosylated and sialylated poly LacNAc glycans.[112] In this study, a hexasaccharide precursor containing $GlcNH_2$, GlcNHTFA, and GlcNHBoc residues was chemically synthesized, in which unnatural $GlcNH_2$, GlcNHTFA, and GlcNHBoc moieties were easily converted into natural GlcNAc. Recombinant fucosyl transferases Hp39-FT and FUT5 recognized LacNAc and LacNHTFA as acceptors for α-1,3 fucosylation, but not $LacNH_2$ and LacNHBoc. By modifying the $LacNH_2$ and LacNHBoc moieties before and after enzymatic fucosylation and sialylation, a library of structurally diversified poly LacNAc glycans was obtained (Fig. 5c).

Recently, Boons *et al.* found that the LacNHBoc moiety in linear poly LacNAc glycan can prevent the branched glycosylation of the proximal Gal residue by β-1,6-*N*-acetylglucosaminyltransferase 2 (GCNT2).[113] This allows for the controlled synthesis of branched poly LacNAc glycans. GlcNHBoc can be galactosylated by bacterial galactosyltransferases, such as recombinant β-1,4-galactosyltransferase from *Helicobacter pylori* (HpGalT) and β-1,3-galactosyltransferase from *Chromobacterium violaceum* (CvGalT), and LacNHBoc cannot be recognized by fucosyltransferase. Lin and coworkers proposed a solution to bypass complex chemical glycosylations when preparing regioselective fucosylated poly LacNAc glycans (Fig. 5d).[114]

Human milk oligosaccharides (HMOs)

HMOs are usually used as metabolic substrates of probiotics to facilitate the formation of the neonatal gut microbiome.[118,119] HMOs may also serve as soluble decoys for viruses and parasites to prevent infants from being infected.[120,121] Because of their structural complexity, access to structurally well-defined HMOs is a major obstacle to elucidating their biological roles and achieving their scaled-up applications. The generation of complex HMOs begins with lactose, and the backbone is elongated by LacNAc, in which the terminal or internal LacNAc moieties are often produced by fucosylations or sialylations.[119,122] Inspired by this repertoire, various HMOs have been synthesized *via* chemoenzymatic methods.[123,124]

In 2015, Cao and coworkers reported the synthesis of lacto-*N*-tetrasaccharide and sialyl lacto-*N*-tetrasaccharides using the OPME method.[125] In this study, the glycosyl acceptor lacto-*N*-tetrasaccharide-proN_3 was first chemically synthesized. Thereafter, sialic acid was installed in the nonreducing end of lacto-*N*-tetrasaccharide-proN_3 to form sialyl lacto-*N*-tetrasaccharides, leveraging the OPME method. Wang and coworkers developed a core synthesis/enzymatic extension strategy to synthesize diverse HMOs.[115] Taking advantage of oligosaccharyl thioether and oligosaccharyl bromide donors, three core structures were chemically prepared using a convergent coupling strategy. Subsequently, these core structures were extended and further decorated by different GTs to create a glycan library of 31 HMOs (Fig. 6a). Despite the OPME strategy significantly simplifying the synthesis procedures, excessive chemical manipulations still limit the synthesis of HMOs. To address this issue, Chen's group attempted to enzymatically assemble lacto-*N*-tetrasaccharide-proN_3 by recombinant GT. They identified a highly productive β-1,3-galactosyltransferase from *C. violaceum*, which exhibited high-efficiency catalysis toward lacto-*N*-triose.[126]

α-1,3/4-Fucosyltransferase from *Helicobacter pylori* DSM 6709 (FucTIII) was expressed and ingeniously applied by Yu and coworkers to prepare several fucosylated HMOs.[127] In this study, FucTIII was incorporated into a sequential one-pot enzymatic strategy to produce a series of fucosylated HMOs, in which Lewis antigen structures, such as Lewis X, Lewis A, and sialyl Lewis X, were included. Furthermore, the enzyme kinetics assay indicated that FucTIII is preferred for the recognition of type II *N*-acetyl lactosamine (LacNAc) as the acceptor, and the catalytic efficiency of FucTIII can be increased by the addition of GlcNAc at the nonreducing end of the acceptor.

In 2021, Yu and coworkers proved that a sulfo-fluorous tag (SF17) added to the reducing end of lactose enables site-selective enzymatic fucosylation of the HMO core (Fig. 6b).[116] SF17-tagged HMO acceptors have the following advantages: (1) enabling site-selective fucosylation by wild-type α-1,3/4-fucosyltransferase from *Bacteroides fragilis* (Bf13FT) and α-1,3/4-FucT from *H. pylori* UA948, (2) enabling selective α-2,6-sialylation on the terminal Gal of the acceptor by wild-type Pd2,6ST, which usually leads to multi-sialylation for the tag-freed or alkyl chain-tagged HMO acceptors, and (3) increasing the catalytic efficiency of β-1,3-galactosyltransferase (β1,3-GalT) from *E. coli* O55:H7 (WbgO), β-1,3-GlcNAcT, and Bf13FT. These strategies fully demonstrate that GTs from bacteria are powerful tools in the field of HMO synthesis.

Human GTs are also available to prepare HMOs. Boons and coworkers systematically described the synthesis of asymmetrical multi-antennary HMOs using a limited number of human GTs.[117] In this study, lactose, bearing the hydrophobic coumarin moiety, acted as a starter to prepare tetrasaccharide LN*n*T using human β-1,3-*N*-acetylglucosaminyltransferase 2 and B4GalT1 in the presence of UDP-GlcNAc and UDP-Gal, respectively. Subsequently, the involvement of GCNT2 enabled

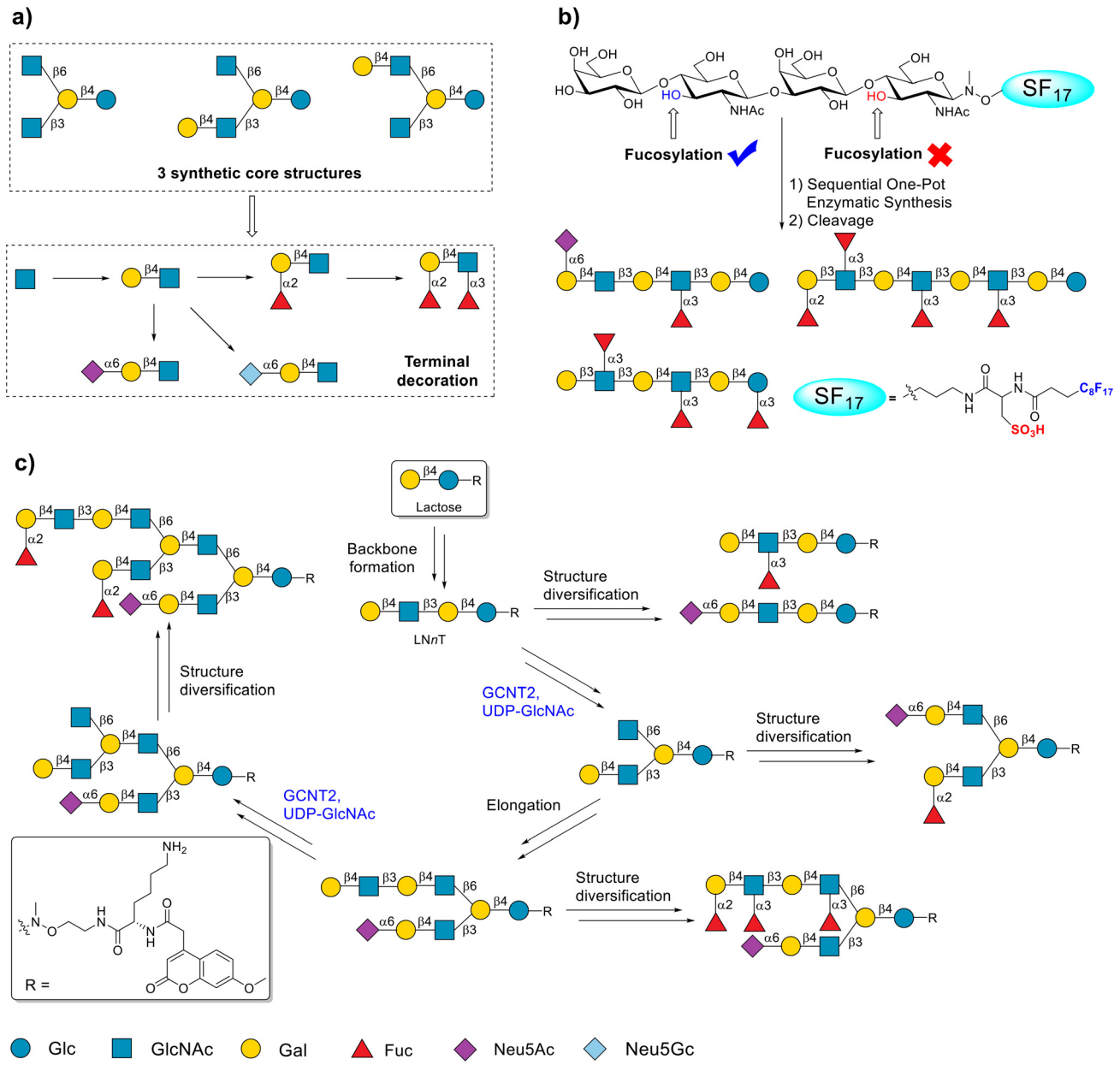

Fig. 6 Chemoenzymatic strategies for the synthesis of HMOs. (a) The core synthesis/enzymatic extension (CSEE) method for synthesis of HMOs. Reproduced with permission from Xiao *et al.*[115] Copyright 2016 American Chemical Society. (b) Acceptor-mediated regioselective synthesis of HMOs. Reproduced with permission from Huang *et al.*[116] Copyright 2021 American Chemical Society. (c) Chemoenzymatic synthesis of asymmetrical multiantennary HMOs. Reproduced with permission from Prudden *et al.*[117] Copyright 2017 Proceedings of the National Academy of Sciences.

the linear tetrasaccharide LN*n*T to produce a branching GlcNAc structure at the internal position, which is the first step in generating multi-antennary asymmetric HMOs. Thereafter, a panel of human GTs (galactoside α-1,2-fucosyltransferase 1, lactosamine α-1,3/4-fucosyltransferase 3, and α-2,6-sialyltransferase 1) was selected to further modify asymmetrical multi-antennary HMO backbones. Finally, a glycan library containing 60 asymmetric, multi-antennary HMOs was constructed and applied in a glycan microarray to probe the interaction of HMOs and several glycan-binding proteins (Fig. 6c). Recently, Fang and coworkers advantageously utilized the expression of GCNT2 in *Pichia pastoris*, and reported an enzymatic modular strategy that produced a library of branched HMOs.[128]

Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) are a family of common linear anionic polysaccharides with specific sulfation patterns. They are composed of alternating hexosamine and uronic acid or galactose residues.[129–131] According to the disaccharide repeat-

ing units of each subtype of GAGs, they are classified into four species: hyaluronic acid (HA), which consists of -β4GlcA-β3GlcNAc1- repeating units; heparin/heparan sulfate (HP/HS), which contains either $(-\alpha4IdoA-\alpha4GlcA1)_n$- or $(-\alpha4GlcA-\alpha4IdoA)_n$-α4GlcNAc- with different sulfation patterns; chondroitin sulfate/dermatan sulfate (CS/DS), which is composed of -β4GlcA/IdoA-β3GalNAc1- repeating units with different sulfation patterns; and keratan sulfate (KS), which is poly-*N*-acetyllactosamine $(-\beta3Gal-\beta4GlcNAc1)_n$- with sulfation at the 6-hydroxy positions.[131,132]

Compared with other polysaccharides, GAGs exhibit substantial market value in medical and cosmetic drug development. For example, hyaluronan is used for arthritis and cosmetic applications,[133] and heparin is a traditional anti-thrombosis agent.[134] In particular, the discovery of the inhibitory effect of GAGs on severe acute respiratory syndrome coronavirus 2 stimulated the design and development of GAG-based antiviral drugs.[135] However, limited access to GAGs, especially homogeneous GAGs, remains, which hinders the achievement of scaled production. The chemoenzymatic approach is thought to be a promising strategy for the large-scale production of GAGs.

A soluble microbial hyaluronan synthase from *Pasteurella multocida* (PmHAS) was used to catalyze GlcA and GlcNAc polymerization to form a hyaluronan chain. PmHAS contains two distinct GT domains that can recognize UDP-GlcNAc and UDP-GlcA as donors.[136,137] Therefore, with the availability of UDP sugar precursors, the elongation of HA only requires one enzyme (PmHAS), facilitating the sequential synthesis of HA.[138,139] Because of the uneconomical use of sugar nucleotide precursors, further studies focused on exploiting the OPME system to produce the desired HA.[140,141] OPME systems require inexpensive monosaccharide modules (GlcNAc and Glc or GlcA) as substrates, rather than expensive sugar nucleotide precursors that provide the substrates. For example, using inexpensive sucrose and GlcNAc as substrates, Elling and coworkers described an economic OPME method to synthesize high-molecular-weight (HMw)-HA, in which the UDP-GlcA and UDP-GlcNAc can be *in situ* regenerated (Fig. 7a).[141]

The chemoenzymatic synthesis of HP/HS depends on the bi-functional enzymes from *Pasteurella multocida* PmHS1 and PmHS2,[142] or a combination of KfiA and KfiC obtained from the *E. coli* K5 strain.[143] A panel of sulfotransferases, including 6-*O*-sulfotransferase (6-OST), 3-*O*-sulfotransferase (3-OST), and *N*-sulfotransferase (NST), contributes to further diversity in the structure of HP/HS. Liu and coworkers reported a simple chemoenzymatic strategy for the tailored synthesis of two structurally homogeneous ULMW heparins (Fig. 7b).[53] In this study, a disaccharide was used as a starting substrate, which was elongated using PmHS2 and KfiA. An unnatural glycosyl donor called UDP-GlcNHTFA was recognized by KfiA, and thus, GlcNHTFA was inserted into the backbone of the target molecule. The GlcNHTFA moiety was converted to $GlcNH_2$ for further *N*-sulfation. Taking advantage of this property, synthetic hexasaccharide and heptasaccharide intermediates were further sulfated at specific sites using C5-epimerases and 2/3/6-OST to accomplish the preparation of two ULMW heparins. Many other efforts have resulted in the cost-effective, controlled, and even gram-scaled synthesis of structurally well-defined HP/HS,[144–147] and HP/HS derivatives and mimetics.[57,148,149]

CS and DS have been widely investigated in chemoenzymatic synthesis for their promising anticoagulant properties.[152,153] Using GT from the *E. coli* K4 strain (KfoC), which can transfer both GalNAc and GlcA from UDPGalNAc and UDP-GlcA to the acceptor to form CS, Liu and coworkers synthesized a library of 15 different CS glycans covering different sulfation pattern glycans, from trisaccharides to nona-saccharides.[154] This group has reported the chemoenzymatic synthesis of seven structurally homogeneous HS and CS chimeras, in which a CS heptasaccharide domain on the reducing end was coupled with an HS pentasaccharide domain on the nonreducing end.[155]

Recently, Huang and coworkers developed a solid phase-supported strategy for the successful chemoenzymatic synthesis of CS proteoglycans (CSPG) for the first time.[150] In this study, Sepharose beads were used as the solid phase support, and were attached to the terminal of peptide substrates using a cleavable linker. Subsequently, the first saccharide xylose was transferred to the peptide substrates using xylosyl transferase-1 to form the initial glycopeptides. The initial glycopeptides were sequentially elongated by β-1,4-galactosyl transferase 7, β-1,4-galactosyl transferase 6, and β-1,3-glucuronic acid transferase 3 (β3GAT3) to form tetra-saccharide-bearing glycopeptides. The phosphorylation of xylose 2-*O* in glycopeptides is required for the catalysis of β3GAT3 (Fig. 7c). Finally, enzymatic chain extension and sulfation result in the preparation of different CSPG glycopeptides. Different from CS, the chemoenzymatic synthesis of DS from chondroitin requires C5-epimerization by epimerase (DS-ep) and sulfation by 4-*O* sulfotransferase (D4ST1).[156]

KS consists of LacNAc repeating units with sulfation occurring on the 6-OH of GlcNAc and Gal. Although KS is involved in many biological events, poor efforts have been made to synthesize structurally well-defined KS glycans. One method for synthesizing KS glycans uses transglycosylation of mutant keratanase II to install the sulfated glycan moieties to produce KS glycans. Ohmae and coworkers efficiently synthesized several fucosylated and sialylated KS glycans using oxazoline donors and mutant keratanase II.[30,157] Another approach involves the sulfation of poly LacNAc glycans by sulfotransferase to produce KS glycans.

Recently, Boons and coworkers reported the use of recombinant human GlcNAc-6-*O*-sulfotransferase (CHST2) and keratin sulfate Gal-6 sulfotransferase (CHST1) for chemoenzymatic synthesis of KS glycans.[158] Their study indicated that CHST2 only sulfates the terminal GlcNAc moieties of LacNAc oligosaccharides, and CHST1 can sulfate the internal Gal moieties in LacNAc chains rather than the terminal Gal.[158] Further work uncovered detailed substrate specificities of CHST1 and also confirmed that α-1,3-fucosylated LacNAc cannot be accepted by CHST1, and Gal-6-*O* sulfation of LacNAc hinders enzymatic α-1,3-fucosylation (Fig. 7d).[151] Through these discoveries, a range of KS glycans with different sulfation and fucosylation patterns was synthesized.

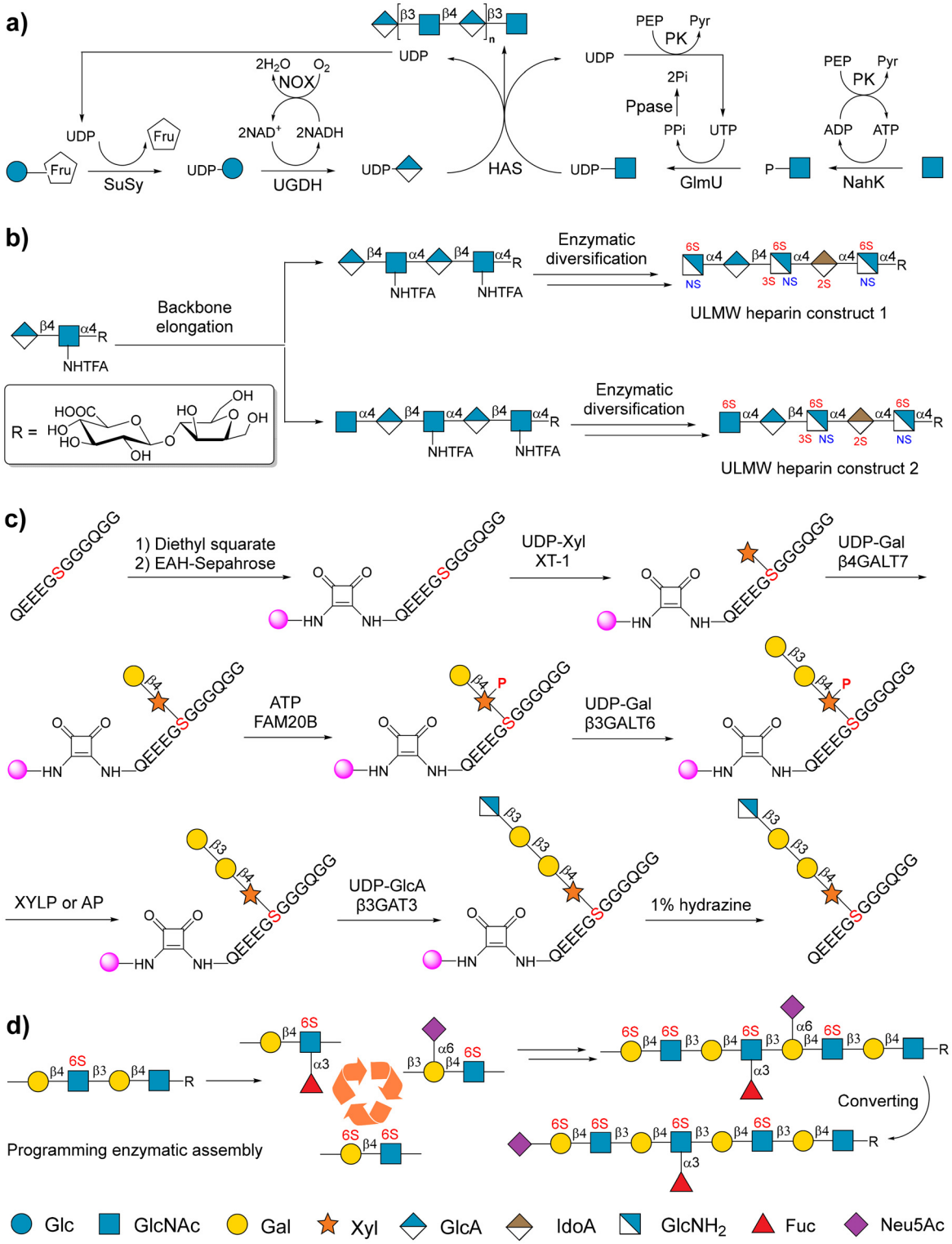

Fig. 7 Chemoenzymatic strategies for the synthesis of GAGs. (a) OPME chemoenzymatic synthesis of HA. SuSy: sucrose synthase from potato. UGDH: UDP-glucose dehydrogenase from *Streptococcus zooepidemicus*. NOX: nicotinamide adenine dinucleotide (NADH) oxidase. NahK: *N*-acetylhexosamine-1-kinase from *Bifidobacterium longum*. PK: pyruvate kinase from the rabbit. GlmU: UDP-GlcNAc pyrophosphorylase *Streptococcus zooepidemicus*. PPase: pyrophosphatase from yeast. HAS: hyaluronan synthase from *Pasteurella multocida*. Reproduced with permission from Eisele *et al.*[141] Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Chemoenzymatic synthesis of homogeneous ultra-low molecular weight (ULMW) HP. Reproduced with permission from Xu *et al.*[53] Copyright 2011, American Association for the Advancement of Science. (c) Solid-phase-supported chemoenzymatic synthesis of CSPG. Reproduced with permission from Lin *et al.*[150] Copyright 2024 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (d) Programming the chemoenzymatic synthesis of KS. Reproduced with permission from Wu *et al.*[151] Copyright 2024 American Chemical Society.

Summary and outlook

In the past few decades, numerous critical physiological or pathological processes involving glycans have been revealed. These findings urgently require glycochemists to prepare structurally well-defined glycans for further exploration of their biological functions. The chemoenzymatic strategy is a powerful tool for preparing a range of biologically related complex glycans, and even glycoconjugates, such as glycopeptides, glycoproteins,[31] and glycolipids.[159,160] Enzymatic glycosylations combined with chemical handles will circumvent tedious protecting group manipulation, and will also increase the regio- and stereo-selectivity of glycosylation. However, at least two factors limit the development of this methodology.

The first factor is the limited availability and poor understanding of GTs. To address this, different protein expression systems have been constructed to produce large-scale GTs, such as *E. coli* expression systems that can efficiently yield a series of bacterial GTs, and an insect expression system that overexpresses many mammalian GTs. Furthermore, crystal structure and mutagenesis studies are promising approaches to increase our understanding of GTs and provide the desired GTs for synthesis.

The second factor is the time-consuming and inefficient reaction post-processing procedures. For example, the synthesis of a deca-saccharide theoretically requires at least nine enzymatic reaction steps, and therefore, at least nine isolation and purification procedures need to be performed. To address this, machine-driven automated enzymatic synthesis has been developed to provide easy access to synthesize complex glycans.[161,162] In addition, many state-of-the-art techniques and tools, such as ultra-performance liquid chromatography,[163] high-resolution mass spectrometry,[164] nanopore sequencing,[165–167] and machine learning,[168] are facilitating the identification and structural analysis of complex glycans, which will be helpful for the rational designing of chemoenzymatic strategies in glycan synthesis. In the future, general protocols will enable the synthesis of glycans that will be as easy as the PCR amplification of DNA.

Author contributions

All authors contributed equally to the manuscript.

Data availability

Data availability is not applicable to this article.

Conflicts of interest

There are no conflicts to declare.

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