Strategies for Automated Enzymatic Glycan Synthesis (AEGS)

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ABSTRACT

Glycans are the most abundant biopolymers on earth and are constituents of glycoproteins, glycolipids, and proteoglycans with multiple biological functions. The availability of different complex glycan structures is of major interest in biotechnology and basic research of biological systems. High complexity, establishment of general and ubiquitous synthesis techniques, as well as sophisticated analytics, are major challenges in the development of glycan synthesis strategies. Enzymatic glycan synthesis with Leloir-glycosyltransferases is an attractive alternative to chemical synthesis as it can achieve quantitative regio- and stereoselective glycosylation in a single step. Various strategies for synthesis of a wide variety of different glycan structures has already be established and will exemplarily be discussed in the scope of this review. However, the application of enzymatic glycan synthesis in an automated system has high demands on the equipment, techniques, and methods. Different automation approaches have already been shown. However, while these techniques have been applied

Abbreviations: AEGS, automated enzymatic glycan synthesis; AEC, anion-exchange chromatography; Asn, asparagine; CAZy, carbohydrate-active enzymes; Cbz, carboxybenzyl; CE, capillary electrophoresis; CE-FLR, capillary electrophoresis with fluorescence detection; (x)CGE-LIF, (multiplexed) capillary gel electrophoresis with laser-induced fluorescence; CFPS-GpS, cell-free protein synthesis driven glycoprotein synthesis; CFMS, compartmented flow microreactor system; FSPE, fluorous solid-phase extraction; DAEase, D-allulose 3-epimerase; DEAE, diethyl aminoethyl; DTT, dithiothreitol; EM, enzyme module; EMR, enzyme membrane reactor; ESI-Q-ToF, electrospray ionization-quadrupole-time-of-flight; FucT, fucosyltransferase; α1,3-FucT, alpha1,3 fucosyltransferase; α3GalT, alpha1,3 galactosyltransferase; β4GalT, beta1,4 galactosyltransferase; GAGs, glycosaminoglycans; GDP-Fuc, guanosine-5′-diphospho-L-fucose; GDP-Man, guanosine-5′-diphospho-D-mannose; GBPs, glycan-binding proteins; GlycoPRIME, glycosylation pathway assembly by rapid in vitro mixing and expression; β3GlcAT, beta1,3 glucuronosyltransferase; GlcNAc, *N*-acetyl D-glucosamine; GM, glycidyl methacrylate; GT-A, glycosyltransferase family A; GT-B, glycosyltransferase family B; GTs, glycosyltransferases; GT-EM, Leloir-glycosyltransferase enzyme module; His_6-tag, $histidine_6$-tag; HILIC, hydrophilic interaction chromatography; HILIC-FLR, hydrophilic interaction chromatography with fluorescence detection; HMOs, human milk oligosaccharides; HNK-1, human killer cell epitope 1; HPAEC, high-performance anion-exchange chromatography; HP/HS, heparin/heparan sulfate; HPLC, high-performance liquid chromatography; IMER, immobilized microfluidic enzyme reactors; IM-MS, ion-mobility mass spectrometry; IR, InfraRed; KS, keratan sulfate; LC-MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; LacNAc, *N*-acetyllactosamine; LC, liquid chromatography; mABs, monoclonal antibodies; MALDI-MS, Matrix-assisted laser desorption mass spectrometry; MEBs, magnetic enzyme beads; MNHS, methacrylic acid *N*-hydroxysuccinimide ester; MS, mass spectrometry; Ni^{2+}-IDA, Ni^{2+}-imino-di-acetic-acid; Ni^{2+}-NTA, Ni^{2+}-nitrilo-tri-acetic-acid; NHS, *N*-hydroxysuccinimide; NS-EM, nucleotide sugar enzyme module; N(*M*)DP-sugar, nucleoside(mono)diphosphate sugar; NMR, Nuclear magnetic resonance; NP, normal phase; OPME, one-pot-multienzyme; OSTs, oligosaccharyl transferases; PAT, process analytical technology; PDSM, pyridyl disulfide ethyl methacrylate; PmHS2, *Pasteurella multocida* heparosan synthase 2; PGC-LC, porous graphitized carbon liquid chromatography; PVCL, poly-*N*-vinylcaprolactam; RAFT, reversible addition–fragmentation chain transfer; RP, reversed phase; Ser, serine; Spy, *Streptococcus pyogenes*; SNFG, Symbol Nomenclature for Glycans (SNFG); STY, space-time-yield; Thr, threonine; TTN, total turnover number; UDP-Gal, uridine-5′-diphospho-D-galactose; UDP-GalNAc, uridine-5′-diphospho-*N*-acetyl-D-galactosamine; UDP-Glc, uridine-5′-diphospho-D-glucose; UDP-GlcA, uridine-5′-diphospho-D-glucuronic acid; UDP-GlcNAc, uridine-5′-diphospho-*N*-acetyl-D-glucosamine; UDP-ManNAc, uridine-5′-diphospho-*N*-acetyl-D-mannosamine; UDP-Xyl, uridine-5′-diphospho-D-xylose; UMP, uridine-5′-monophosphate; VP, *N*-vinylpyrrolidone; VPI, *N*-vinylpiperidone; VCL, *N*-vinylcaprolactam.

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for several glycans, only a few strategies are able to conserve the full potential of enzymatic glycan synthesis during the process – economical and enzyme technological recycling of enzymes is still rare.

In this review, we show the major challenges towards Automated Enzymatic Glycan Synthesis (AEGS). First, we discuss examples for immobilization of glycans or glycosyltransferases as an important prerequisite for the embedment and implementation in an enzyme reactor. Next, improvement of bioreactors towards automation will be described. Finally, analysis and monitoring of the synthesis process are discussed. Furthermore, automation processes and cycle design are highlighted. Accordingly, the transition of recent approaches towards a universal automated glycan synthesis platform will be projected. To this end, this review aims to describe essential key features for AEGS, evaluate the current state-of-the-art and give thought- encouraging impulses towards future full automated enzymatic glycan synthesis.

1. Introduction

Monosaccharides belong to the diverse class of carbohydrates and do not only function as the main energy source for living organisms, but also serve as building blocks for oligo- and polysaccharide structures that appear in all kinds of animals, microorganisms, and even viruses (Varki et al., 2022). These glycans range from simple linear chains to highly complex branched molecules and are species-, tissue- and cell-specific. Presented on the surface of eukaryotic or prokaryotic cells, and viruses, glycans play important roles in essential physiological processes, like cell-cell interactions, viral and bacterial attachment, cancer evasion and metastasis, and immune response (Varki, 2017). Attached to membrane-bound glycoproteins and -lipids or secreted glycoproteins, they are crucial for their proper function and mediation of intra- and extracellular processes (Shivatare et al., 2022). Defects in the glycosylation patterns of glycoproteins have been connected with several human diseases (Magalhães et al., 2021; Miura and Endo, 2016; Smith and Bertozzi, 2021). Regarding these functions in essential physiological processes, well-defined tailored glycan structures for therapeutic applications in medicine, ingredients for cosmetics, and nutrition supplements gained importance over the last decade.

Human Milk Oligosaccharides (HMOs) for example play roles in important biological mechanisms like the protection of the gastrointestinal tract against toxins, and viral and bacterial adhesion (Bode, 2012; Chen, 2015). They are also involved in the mediation of the immune response and brain development of infants (Georgi et al., 2013). With over 200 elucidated structures, they display an extremely high structural diversity (Kobata, 2010; Kottler et al., 2013; Sprenger et al., 2017; Thurl et al., 2017). For further studies on the structure and benefits of HMOs, single compounds with a high grade of purity are needed (Baumgartner et al., 2014). Moreover, HMOs can be used as pre- and probiotic supplements in baby formula. To achieve these goals, automated and scalable production of tailored HMOs is needed (Petschacher and Nidetzky, 2016).

For therapeutic glycoproteins, the correct glycosylation is crucial for their effectiveness and safe application without side effects during treatment. Since, for example, the recombinant production of monoclonal antibodies (mAbs) is mostly executed in hosts that do not glycosylate proteins with a human glycosylation pattern, enzymatic post-translational in situ glycosylation and glycan remodeling have gained growing interest over the last few years (Wang et al., 2019).

The group of linear glycosaminoglycans (GAGs), containing the well-known hyaluronic acid (HA), heparin/heparan sulfate (HP/HS), and keratan sulfate (KS), plays also important roles in pathogen defense, coagulation, inflammation, and cell adhesion (Aquino and Park, 2016; Kowitsch et al., 2018; Sobczak et al., 2018). Regarding these functions, they are utilized in therapeutic agents and cosmetic applications. Since today most of the glycosaminoglycans are extracted from animal tissue and therefore often contaminated or not well defined, it is also of high interest to establish enzymatic production processes (Gottschalk and Elling, 2021).

In recent years lipopolysaccharide (LPS)-based glycoconjugates have become promising candidates as vaccines. Despite ongoing preclinical studies it is assumed, that these will be effectively applied against gram-negative bacterial pathogens (Zhu et al., 2021).

Moreover, glyconanoparticles were proven to serve as effective tools for cancer immunotherapy. Here, the immunization of mice with tumor-associated carbohydrate antigens showed promising results (Reuven et al., 2019).

Since the class of galectin binding lectins, the galectins, are known to be deeply involved in cancer development and metastasis (Girotti et al., 2020; Rodrigues et al., 2018), it is thought that modified glycans could act as inhibitors and are therefore interesting candidates for cancer treatment (Heine et al., 2022a; Laaf et al., 2019). Glycan microarrays are a powerful tool to discover and analyze glycan-binding proteins (GBPs) and illuminate their function (Mende et al., 2019; Rillahan and Paulson, 2011; Wisnovsky and Bertozzi, 2022). In the last decade various methods, ranging from chip assays to the establishment of glycan libraries have been developed (Bagdonaite et al., 2022; Campanero-Rhodes et al., 2020; Wisnovsky and Bertozzi, 2022). With these, it is now possible to decipher the glyco-code of glycomes of different organisms.

With all these examples it is clear to see, that the development of different synthesis routes for tailor-made glycan structures for medical, therapeutic, cosmetic, and food applications, nowadays is of particular interest and a topic of research (Gottschalk and Elling, 2021; Malik et al., 2021; Petschacher and Nidetzky, 2016; Rexer et al., 2021; Wang et al., 2019). In general, these approaches can be categorized into three groups: chemical synthesis, enzymatic synthesis, and chemo-enzymatic synthesis (Li et al., 2019c). Chemical glycan synthesis is characterized by complex multi-step reactions under extreme conditions with moderate yields and large amounts of toxic by-products. Via repetitive cycles of selective protection, deprotection, and glycosylation reactions, glycan structures can be selectively synthesized (Guberman and Seeberger, 2019; Panza et al., 2018).

In contrast, enzyme-assisted glycan synthesis is an attractive alternative to chemical synthesis as it has the advantage of achieving quantitative regio- and stereoselective glycosylation in a single step. Although strict substrate specificity appears as a disadvantage, a comprehensive enzyme toolbox for glycosylation reactions and multigram synthesis of nucleotide sugars (Fig. 1) as precursors of glycosyltransferases facilitate the enzymatic synthesis of complex glycans (Brockhausen, 2014; Moremen et al., 2018; Nidetzky et al., 2018; Rexer et al., 2021; Weijers et al., 2008). Chemo-enzymatic synthesis tries to combine the advantages of both approaches into one process by integrating the flexibility of chemical derivatization and the stereoselectivity of enzymes (Li et al., 2019c).

While the biggest challenge of chemical glycan synthesis is the choice of suitable protecting groups and the preparation of selectively protected monosaccharides, that allows stereoselective glycosylation (Wang et al., 2007), the enzymatic synthesis can perform even sterically demanding coupling reactions, that involve sialic acid, selectively and without the need of protecting groups (Wen et al., 2018). Moreover, enzymatic reactions are carried out in aqueous solutions with physiological pH values, and moderate temperatures under natural non-toxic conditions, making them a lucrative environmentally friendly approach. Furthermore, due to their substrate specificity, it is possible to perform enzyme-catalyzed glycan synthesis in one-pot reactions reaching high yields with little equipment effort and only the need for

purification of the final product (Yu and Chen, 2016).

For the enzymatic synthesis of glycans, three classes of carbohydrate-active enzymes (CAZy, Carbohydrate Active Enzymes database, http://www.cazy.org/) (Drula et al., 2021) are relevant: Glycosidases and their derived glycosynthases, non-Leloir glycosyltransferases, and Leloir glycosyltransferases. Glycosidases (EC 3.2) catalyze the hydrolysis of glycosidic bonds in complex glycans. They are classified as *endo*- and *exo*-glycosidases in dependency on their target motif. Both classes can be efficiently used in transglycosylation reactions (Armstrong et al., 2019; Mészáros et al., 2021), however, in most cases with moderate product yields due to competing product hydrolysis. Site-directed mutagenesis at the catalytic site generates glycosynthases lacking hydrolytic activity (Cobucci-Ponzano et al., 2011; Hancock et al., 2006). Glycosyltransferases (GTs, EC 2.4) are the most widely used enzymes for glycan synthesis. They catalyze the transfer of a monosaccharide donor to an acceptor substrate in a high regio- and stereoselective manner and a single step. Non-Leloir GTs use monosaccharide-1-phosphates or other energy-rich disaccharides, like sucrose, as donor substrates. Leloir GTs use nucleotide sugars (Fig. 1) as donor substrates. Hereby, GTs are classified by their reaction mechanism into retaining and inverting enzymes (Fig. 2), depending on whether they retain or invert the anomeric configuration during sugar transfer (Lairson et al., 2008; Nidetzky et al., 2018; Rexer et al., 2021; Wen et al., 2018). Leloir-GTs are classified into the super-families GT-A and GT-B displaying characteristic protein folds. Reactions of the GT-A family are dependent on divalent metal ions that bind to a conserved DXD motif for complexing the nucleotide sugar donor. Catalysis of the GT-B family members is independent of any interaction with metal ions, as activated sugar donors bind to basic amino acids.

With a continuously expanding toolbox of native and engineered glycosyltransferases, and a better understanding of their substrate specificities and reaction mechanisms, the popularity of enzymatic and chemo-enzymatic glycan synthesis is rapidly growing. In a recent study by the DeLisa group a method allowing the recombinant expression of 100 active mammalian and bacterial GTs in *Escherichia coli* hosts was established (Jaroentomeechai et al., 2022). Furthermore, different approaches to high-yielding, cost-efficient processes that synthesize defined glycan structures were developed (Rexer et al., 2021) and can now be expanded due to better availability of GTs. State of the art in enzymatic glycan synthesis includes the sequential utilization of Leloir-GT enzyme modules (GT-EM) coupled to enzyme module systems for (re)generation of nucleotide sugars (NS-EM) in a one-pot reaction. The term one-pot-multienzyme (OPME) synthesis was introduced to indicate that product isolation is not necessary for subsequent GT reactions.

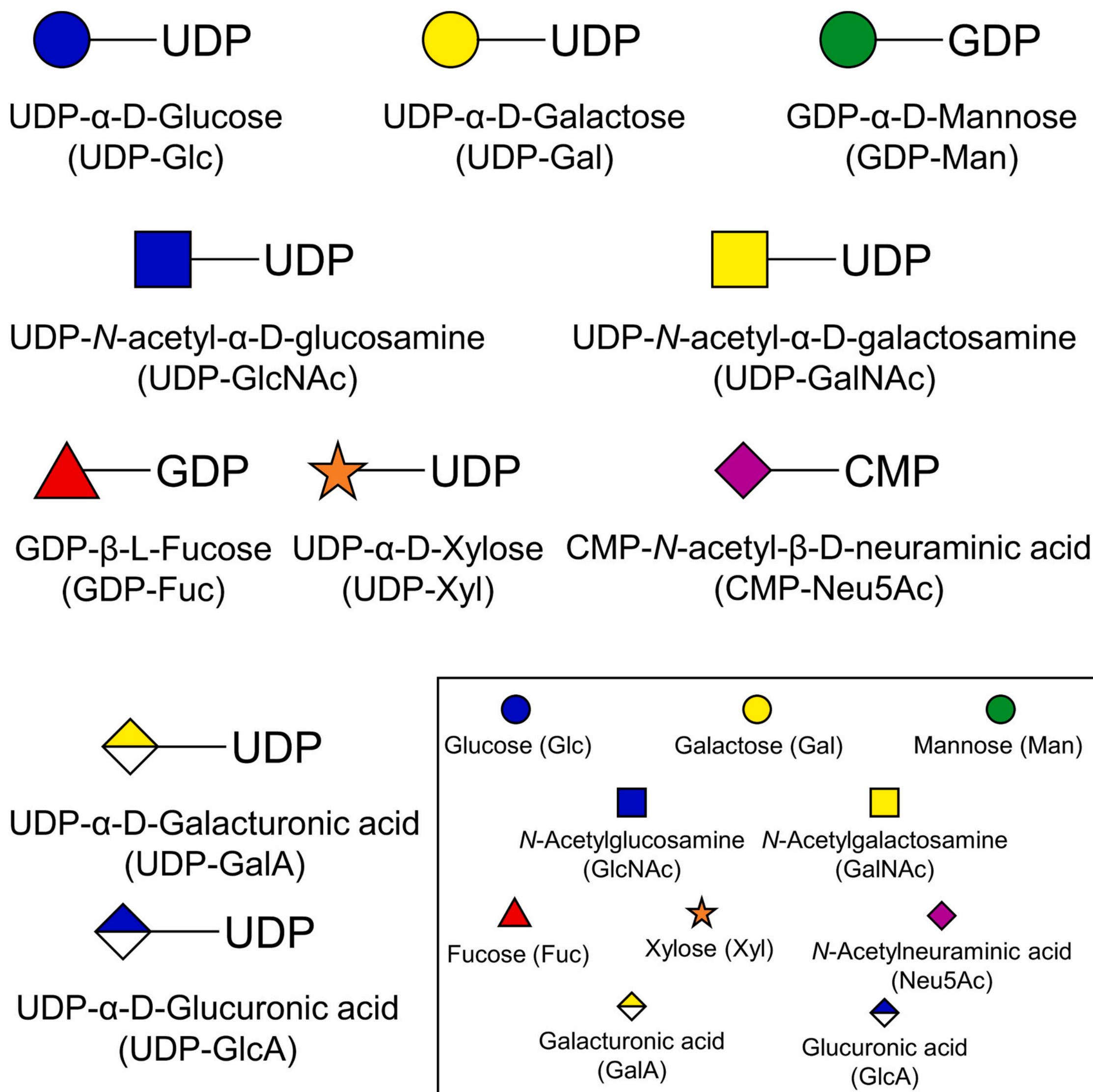


Fig. 1. Most common nucleotide sugars for glycan synthesis. The sugar symbols are depicted according to the Symbol Nomenclature for Glycans (SNFG) (Neelamegham et al., 2019; Varki et al., 2015).

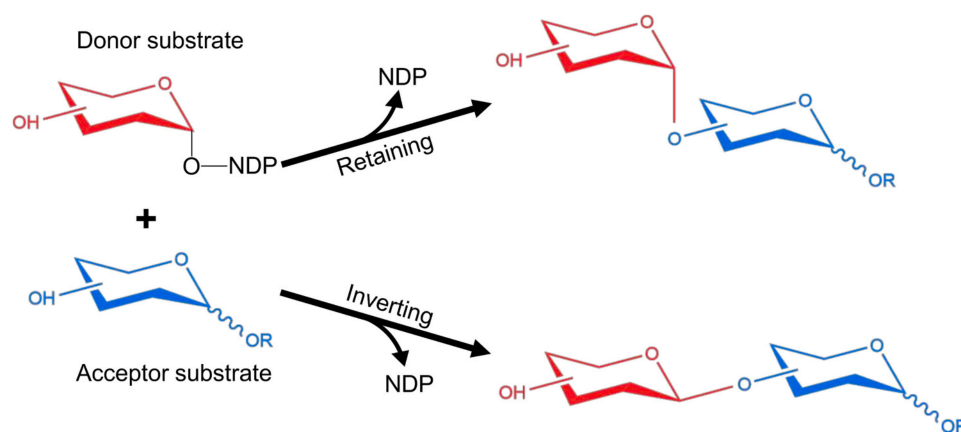


Fig. 2. Reaction mechanisms of glycosyltransferases (GTs). Retaining GTs retain the anomeric configuration of the donor substrate (α-anomer as example) while inverting GTs invert the anomeric configuration (α- to β-anomer as example).

NS-EMs consist of enzyme cascades of salvage pathways including sugar kinases, N(*M*)DP-sugar pyrophosphorylases, and a pyrophosphatase (Engels and Elling, 2016; Frohnmeyer and Elling, 2023) and for bio-economic synthesis, NS-EMs were further extended by co-factor regenerating enzymes (Frohnmeyer et al., 2022; Mahour et al., 2022; Zheng et al., 2022).

Examples of products from one-pot batch synthesis are HMOs (McArthur et al., 2019), blood and Lewis group antigens (Ye et al., 2019), hyaluronic acid and sulfated glycosaminoglycans (GAGs) (Eisele et al., 2018; Gottschalk et al., 2022; Gottschalk et al., 2021; Gottschalk and Elling, 2021; Gottschalk et al., 2019), and glycosphingolipids (GSL) (Li and Guo, 2021).

In conclusion, having an enzyme toolbox at hand, the number of GTs and their catalytic efficiency determines the reachable complexity of glycans. Automation shall be the next advanced step for customized enzymatic glycan synthesis.

The utilization of nucleotide sugars as donor substrates is an effective cost factor when it comes to large-scale or automated enzymatic glycan synthesis. Bio-economic access to nucleotide sugars is therefore a prerequisite. Enzyme cascades for in situ regeneration of N(*M*)DP-sugars have been developed, however, integration into automated glycan synthesis is complicated by additional enzymes. Instead, production on larger scales starting from simple monosaccharide building blocks has to be implemented for a constant supply. In recent studies, multi-gram-scale productions of several nucleotide sugars have been established (Fischöder et al., 2019; Frohnmeyer and Elling, 2023; Frohnmeyer et al., 2022; Li et al., 2021; Mahour et al., 2022; Wang et al., 2022).

In this review, challenges to the use of glycosyltransferases in automated enzymatic glycan synthesis will be summarized and discussed. The overall challenge for fast substrate conversion in each step is to define the optimal conditions for the GT cascade to reach high product yields and total turnover numbers (TTN, $g_{Product}/g_{Enzyme}$).

Accordingly, this review focuses on the enzyme technological aspects that are required for an economical establishment of Automated Enzymatic Glycan Synthesis (AEGS): Immobilization of glycans or enzymes, incorporation in a bioreactor, and glycan analysis. We will summarize and discuss the different operation strategies in automated enzymatic synthesis. Recent reports focused on substrate immobilization as well as 'catch-and-release' for product recovery (Li et al., 2019a; Sears and Wong, 2001; Wen et al., 2018). The GTs are denatured or otherwise discarded after the reaction, leading to unnecessarily high costs. We will therefore focus on strategies including enzyme immobilization for their reuse. Moreover, we take a closer look at the modularization and compartmentalization of enzyme reactors. These kinds of reactors enable changes of reaction routes, a high variability of educt and buffer streams, and fast exchanges of enzyme modules for a higher product flexibility, but maintain the concept of an automated process.

Finally, modes of glycan analysis are outlined, and their usability in fully automated glycan synthesis processes is assessed.

2. 'Mode of Operation' for automated enzymatic glycan synthesis

Among all large groups of biopolymers – namely proteins, nucleic acids, and glycans – the latter are the most abundant on earth (Varki, 2017). While automated solid-phase synthesis is established and widely applied for peptides, DNA, and RNA, commercial solutions for the production of oligosaccharides are still rare. Although several strategies and systems are described, the holy grail for glycan synthesis to line up with the big inventions for the automated production of biopolymers is still not found. In this chapter we want to discuss crucial basic criteria of automated glycan synthesis, introducing different approaches and giving an outlook on promising technical devices.

Already in 2001 Seeberger and colleagues proposed essential key features for the automated, chemical synthesis of oligosaccharides (Plante et al., 2001), which also inspire modes for automated enzymatic synthesis. For such device it is mandatory that (a) chemical manipulations are performed in iterative cycles at variable temperatures; (b) the growing oligosaccharide chains are attached to a solid support for cycle maintenance and easy purification; (c) all involved support structures are chemically inert to all conditions during the synthesis cycles but can be removed efficiently from the final product; (d) functional groups that are not involved in the formation of the new glycosidic bond are protected by protection groups, and (e) glycosylation reactions are efficient and stereospecific, leading to high product yields. With their proposed key features and objectives, the group of Seeberger optimized the chemical automated glycan assembly during the last years, achieving oligosaccharides of up to 150 sugar units in their commercially available glycan synthesizer Glyconeer 2.1 (Hahm et al., 2017; Joseph et al., 2020; Lin et al., 2022; Plante et al., 2001). Here, a solid support resin with a linker is mixed with monosaccharide building blocks. Assembly of oligosaccharides is achieved in repetitive, computer-controlled reaction cycles of consecutive deprotection, glycosylation, and wash steps with renewed protection. Removal of selected temporary protection groups initiates a new glycosylation cycle by allowing a nucleophilic attack of the new building block. The final oligosaccharide is cleaved off and released from the solid phase resin and the remaining protecting groups are removed (Hahm et al., 2017).

While chemical glycan assembly with the Glyconeer 2.1 is important pioneering work towards automation, chemical synthesis harbors several challenges and issues in contrast to enzymatic synthesis. Formation of stereo- and regioselective glycosidic bonds is only possible

with elaborated techniques that shield one part of the transcending oligosaccharide for formation of either α- or β-anomers (Plante et al., 2001). Glycosyltransferases, on the other hand, catalyze stereo- and regioselective glycosylations without any further intervention. Furthermore, chemical synthesis of oligosaccharides often includes an anhydrous environment and tightly controlled protecting, deprotecting, and glycosylation steps, while enzymatic reactions can occur under mild, aqueous conditions without toxic byproducts (Hsu et al., 2011; Wen et al., 2018).

Despite all differences in reaction conditions and environment, both, automated chemical and enzymatic synthesis have a strong dependence on solid-phase support in common. While chemical synthesis relies on the attachment of the synthesized oligosaccharide to the solid phase, for enzymatic synthesis, immobilization of either the acceptor substrate or the glycosyltransferase, is used (Fig. 3).

In the following sections, we discuss enzymatic strategies for solid phase synthesis and synthesis by catch-and-release of glycans. We emphasize also glycan synthesis with immobilized enzymes with the potential for integration into automated glycan synthesis. We describe the mode of immobilization as depicted in Fig. 5, Fig. 6, and Fig. 8), and show selected glycan structures of the respective publications in Fig. 4 and Fig. 7). For more detailed information, more glycan structures are listed in Table 1-3.

2.1. Solid-phase synthesis: immobilized substrates and soluble glycosyltransferases

Enzymatic glycan synthesis with glycosyltransferases in solution has been applied for the synthesis of a variety of glycan structures (Fig. 4). Different strategies have been developed using immobilized substrates: solid-phase- or polymer-bound (Fig. 5) or tagged substrates for capture and release of products (Fig. 6).

2.1.1. Solid phase or polymer-bound synthesis

One strategy of solid phase synthesis is the reversible attachment of the glycan acceptor to Sepharose (Blixt and Norberg, 1997) (Fig. 5A, Table 1). Here, an initial sugar is activated with a thiol group at the reducing end. This sugar was then reacted with thiopropyl Sepharose, resulting in a disulfide bond between sugar and Sepharose. After the enzymatic reaction, the oligosaccharide can be cleaved off the Sepharose by reduction of the disulfide bond with β-mercaptoethanol or dithiothreitol. *N*-iodoacetyl aminopropyl controlled pore glass can also serve as support (Halcomb et al., 1994) (Fig. 5B, Table 1). This resin can be derivatized with a halide-activated acceptor sugar by nucleophilic esterification. After enzymatic glycosylation, the oligosaccharide is released from the solid support via hydrazinolysis (Halcomb et al., 1994). The efficiency of solid-phase synthesis may be limited by substrate accessibility leading to incomplete glycosylation and product mixtures after several cycles.

Studies to overcome the limitations of heterogeneous solid-phase synthesis use temperature-responsive water-soluble polymers (Zhang et al., 2018)(Fig. 5C, Table 1). Being soluble at low temperatures and becoming insoluble at higher temperatures, they combine the benefits of homogeneous enzyme catalysis and recovery of polymer-bound products (Huang et al., 2001). The polymer-bound glycosyl acceptor is glycosylated enzymatically in solution. Increasing the temperature renders the polymer insoluble, and the polymer-bound product can be easily recovered by centrifugation of the solution (Huang et al., 2001). This technique was also applied for automated enzymatic glycan synthesis in a peptide synthesizer by the Wang group (Zhang et al., 2018), a detailed description can be found in chapter 4. A disadvantage may arise from the relatively low temperature of around 25 °C for soluble polymers that can slow down enzymatic reactions in solution and thus favors enzymes with a low optimal reaction temperature.

A further example is the use of globular protein-like poly(amidoamine) dendrimers as polymer support for the initial sugar substrate. A protease- or photocleavage site is used to release the final product from the solid support (Matsushita et al., 2010) (Fig. 5D, Table 1). In a modified High-Performance Liquid Chromatography (HPLC) device performing repetitive cycles of sugar elongation with recombinant glycosyltransferases, the technology was termed an 'Artificial Golgi Apparatus' (Matsushita et al., 2010).

2.1.2. Capture and release strategies

Another elegant way is the "catch and release" strategy for tagged substrates for the capture and release of products in solution. Keeping the substrates unbound in solution overcomes potential steric restrictions that affect enzymatic reactions (Fig. 6, Table 2).

A promising tool for the efficient capture of tagged substrates is "fluorous solid-phase extraction" (FSPE) (Cai et al., 2014; Hwang et al., 2014; Jaipuri and Pohl, 2008) (Fig. 6A, Table 2). Here, the affinity of perfluoro groups to fluorous surfaces and solvents is exploited. The fluorous tag ($CF_3(CF_2)_n$) is attached to the glycosyl acceptor before the enzymatic glycosylation reaction (Yang et al., 2010). The length of the linker is flexible and has an influence on binding, reaction efficiency, and solubility of the tagged glycan and should therefore be carefully selected (Hwang et al., 2014). After glycosylation, the solution is loaded on an FSPE column and non-fluorous compounds are washed away, resulting in a pure fluorous-tagged glycan product (Cai et al., 2014; Hwang et al., 2014; Jaipuri and Pohl, 2008). This strategy was applied for chemical and enzymatic one-pot synthesis (Hwang et al., 2014) (Yang et al., 2010) and automated chemical synthesis (Jaipuri and Pohl, 2008) of diverse glycan structures.

The principle of product capturing by ion exchange resins was also

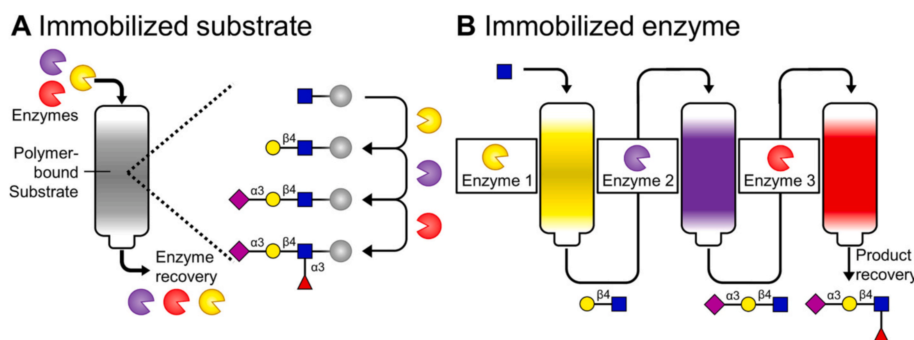


Fig. 3. Strategies towards automated enzymatic glycan synthesis (Sears and Wong, 2001). An exemplary synthesis of sialyl Lewis^x by three glycosyltransferases is depicted. (A) The glycan acceptor is immobilized in the solid phase. Soluble enzymes, buffer, and sugar building blocks are in the liquid phase. Glycosylation is performed on immobilized glycans and soluble compounds are removed after the enzymatic reaction, the elongated glycan is retained in the solid phase. (B) Glycosyltransferases are immobilized on the solid phase. The growing glycan is passed through different reaction modules together with respective buffer and sugar building blocks. Enzyme immobilization allows the performance of multiple cycles of glycosylation using the same enzymes. After final glycosylation, the product is recovered from the flow-through. Immobilized and nascent glycans are shown following Symbol Nomenclature For Glycans (SNFG) (Neelamegham et al. 2019; Varki et al. 2015) (Fig. 1), the solid phase is shown in gray. Different enzymes are shown in yellow, violet, and red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

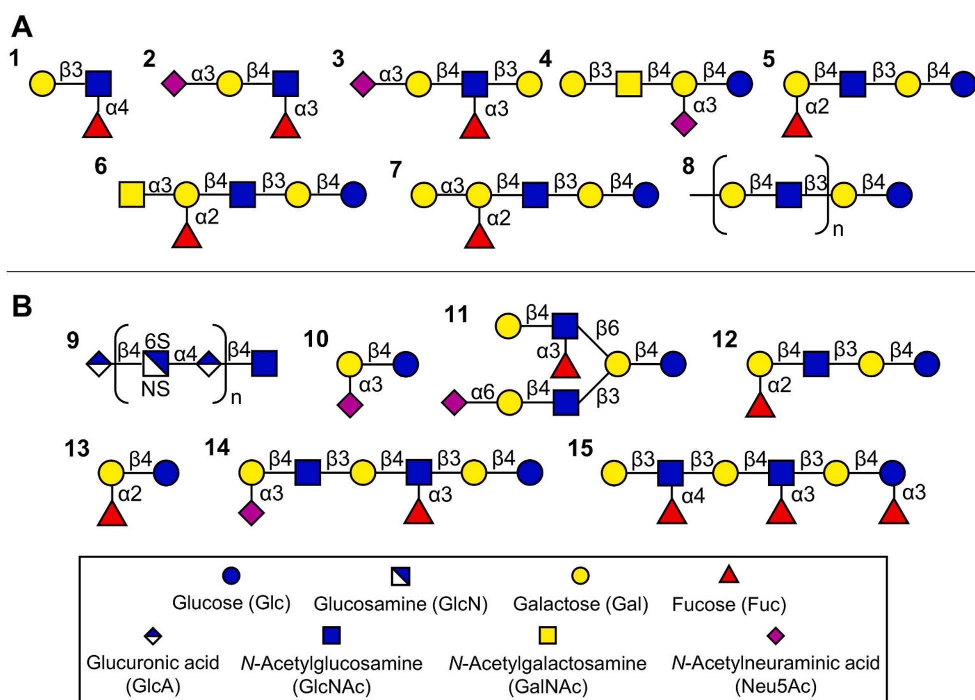


Fig. 4. Exemplary glycan structures of synthesis with immobilized substrates (A) and catch and release driven synthesis (B). 1 Lewis[a] trisaccharide (Blixt and Norberg, 1997), **2** sialyl-Lewis[x] tetrasaccharide (Huang et al., 2001; Matsushita et al., 2010), **3** *Helicobacter pylori* inhibitor oligosaccharide (Halcomb et al., 1994), **4** ganglioside GM1, **5** blood group H-antigen, **6** blood group A-antigen, **7** blood group B-antigen, **8** (Poly-) LacNAc (Zhang et al., 2018), **9** Heparan sulfate oligosaccharide (Cai et al., 2014), **10** sialoside (Hwang et al., 2014), **11** branched HMO (Li et al., 2019a), **12** HMO fucosyl-lacto-*N*-neotetraose (Zhu et al., 2017), **13** 2'fucosyl-lactose, **14** sialylated and fucosylated poly-LacNAc derivative (Fang et al., 2022), **15:** HMO trifucosyl-*para*-lacto-*N*-hexaose II (TF-*p*LNH II) (Bai et al., 2022).

Table 1
Enzymatic glycan synthesis with immobilized substrates.

Glycosyltransferases	Products	Solid Phase	Tag	Process	Reference
• α-1,3/4-Fucosyltransferase (Human milk)	Lewis[a] trisaccharide Fig. 4 Structure 1	Sepharose	Thiol group on aglycone	One-Step, Release via Oxidation	(Blixt and Norberg, 1997)
• β-1,4-galactosyltransferase (Cytel) • α-2,3-sialyltransferase (Cytel)	*H. pylori* inhibitor oligosaccharide Fig. 4 Structure 3	Pore glass		Sequential, Release via hydrazinolysis	(Halcomb et al., 1994)
• β-1,4-galactosyltransferase (bovine milk) • α-1,3-fucosyltransferase (FucT V) • α-2,3-sialyltransferase (α-2,3-sialT)	Sialyl-Lewis[x] tetrasaccharide Fig. 4 Structure 2	Thermo-responsive polymers	*N*-acrylylsuccinamide (NASI) groups	Multistep purification	(Huang et al., 2001)
• β-1,4-galactosyltransferase (LgtB) • β-1,3-galactosyltransferase (LgtA) • α-2,3-sialyltransferase (PmsT1) • β-1,4-*N*-acetylgalactosaminyltransferase (CgtA) • β1,3-galactosyltransferase (CgtB) • α-1,3/4-Fucosyltransferase (FucT) • α-1,3 *N*-acetyl-galactosaminyl-transferase (BgtA) • α-1,3-galactosyltransferase (GTB)	GM1, blood group O, A, and B antigens, and poly-LacNAc Fig. 4 Structure 4–8	Thermo-responsive polymers	Thioether linker oxidized to a sulfone by hydrogen peroxide	Automated in Peptide Synthesizer	(Zhang et al., 2018)
• β-1,4-galactosyltransferase (Human, Toyobo Ltd.) • α-1,3-fucosyltransferase (Human Calbiochem) • α-2,3-sialyltransferase (Rat Calbiochem)	Sialyl-Lewis[x] tetrasaccharide Fig. 4 Structure 2	Poly(amidoamine) dendrimer	Reactive ketone	Automated HPLC	(Matsushita et al., 2010)

developed in different studies (Li et al., 2019a; Zhu et al., 2017). A small aminopropyl linker attached to the acceptor sugar was used for product binding at acidic conditions on a cation-exchange resin. In an acidic milieu, the amino linker forms an ammonium cation, facilitating binding to a cation exchange resin. Adjustment to alkaline pH by ammonium bicarbonate releases the amino linker-tagged glycan from the resin with subsequent vacuum evaporation of the eluent buffer (Zhu et al., 2017) (Fig. 6B, Table 2). Another approach utilizes the interaction of a sulfonate tag with a diethyl aminoethyl (DEAE) anion exchange resin as the capture step and product release by pH adjustment (Li et al., 2019a) (Fig. 6C, Table 2). This technique was used to generate an automated glycan synthesizer and is further described in chapter 4.

Acceptor substrate tagging with hydrophobic carboxybenzyl (Cbz) and subsequent capture with a C18 cartridge was applied for the multistep one-pot multienzyme synthesis of human milk oligosaccharides with high purity (Bai et al., 2022) (Fig. 6D, Table 2). Similar to the previously described thiopropyl Sepharose approach (Blixt and Norberg, 1997), a thiol group linked to the acceptor enables the catch of the product on thiopropyl Sepharose via the formation of disulfide bonds (Fang et al., 2022) (Fig. 5A, Table 2). Supplementation with DTT reduces the bonds and releases the product either for characterization or the next glycosylation cycle. The influence of residual DTT on future

Table 2
Enzymatic glycan synthesis with 'catch and release'of products.

Glycosyltransferases	Products	Solid Phase	Tag	Process	Reference
• α-1,3-*N*-acetylglucosaminyltransferase (KfiA) • heparosan synthase-2 (pmHS2)	Heparan sulfate oligosaccharide Fig. 4 Structure 9	Fluorous solid-phase	FBoc Linker	Sequential	(Cai et al., 2014)
• α-2,3-sialyltransferase (PmST1) • α-2,6-sialyltransferase (Pd2,6ST) • α-1,3-galactosyltransferase (bovine)	Sialosides and Galactosides Fig. 4 Structure 10	Fluorous solid-phase	Fluorous tag	One-pot multienzyme	(Hwang et al., 2014)
• β-1,4-galactosyltransferase (Mammalian B4GalT1) • β-1,3-*N*-acetylglucosaminyltransferase (Mammalian B3GNT2) • *N*-acetyllactosaminide β-1,6-*N*-acetylglucosaminyltransferase (Mammalian GCNT2) • β-galactoside α-2,6-sialyltransferase (Mammalian ST6Gal1) • β-galactoside α-2,3-sialyltransferase (Mammalian ST3Gal4) • α-*N*-acetyl- neuraminide α-2,8-sialyltransferase (ST8Sia1) • α-fucosyltransferases (Mammalian FUT1, FUT3, FUT5) • α-2,3-sialyltransferase (PmST1) • α-2,8-sialyltransferase (CstII) • β-1,4-*N*-acetylgalactosaminyltransferase (CgtA) • β-1,3-galactosyltransferase (CgtB)	Diversified LacNAc multimers, HMOs, gangliosides, refer to publication Fig. 4, Structure 11	Diethylaminoethyl ion exchange resin	Sulfonate tag	Automated dispenser glycosynthesizer	(Li et al., 2019a)
• β-1,3-*N*-acetylglucosaminyltransferase (NmLgtA) • β-1,4-galactosyltransferase (NmLgtB) • α-1,2-fucosyltransferase (Hmα1,2FT) • α-1,3-fucosyltransferase (Hpα1,3FT)	HMOs Fig. 4, Structure 12	Cation Exchange Resin	Amino linker	Sequential	(Zhu et al., 2017)
• β-1,3-*N*-acetylglucosaminyltransferases (NmLgtA and Hpβ3GlcNAcT) • β-1,3-galactosyltransferase (NmLgtB and Cvβ3GalT) • α-1–2-fucosyltransferase (Hm2FT and Te2FT) • α-1–3/4-fucosyltransferase (Hp3/4FT) • α-2–3-sialyltransferase (PmST3) • α-2–6-sialyltransferase (Pd2,6ST)	HMOs Fig. 4 Structure 15	C18 Catridge	Carboxybenzyl group	Multistep one-pot	(Bai et al., 2022)
• α-2,3-sialyltransferase (PmST1) • β-1,4-*N*-acetyl galactosaminyltransferase, (CjCgtA) • β-1,3 galactosyltransferase (CjCgtB) • α-1,2 fucosyltransferase (Hmα1,2FucT) • α-1,3 *N*-acetylgalactosaminyltransferase (HmBgtA) • α-1,3 galactosyltransferase (hGTB) • β-1,3 N acetylglucosaminyltransferase (HpLgtA) • β-1,4 galactosyltransferase (NmLgtB) • α-1,3 fucosyltransferase (Hpα1,3FucT) • α-2,6 sialyltransferase (Pd2,St6)	GM1, ABH, antigens, poly LacNAc Fig. 4 Structure 13–14	Sepharose	Thiol Tag	Sequential	(Fang et al., 2022)

disulfide bond formation was countered by an excess of thiopropyl Sepharose. Importantly this strategy is only viable for glycosyltransferases without crucial disulfide bonds.

Each strategy has its advantages and disadvantages and none of these strategies aims for optimized utilization of the precious biocatalysts (Pohl, 2019). Performing the reaction with the extending oligosaccharide attached to the solid phase, either permanent or transient, requires supplying the soluble glycosyltransferase to the solution. After each catalysis step, the enzyme has to be removed, followed by the addition of the next glycosyltransferase. To avoid wastage of valuable enzymes, recovery can be performed by a technique like affinity chromatography, microfiltration, or precipitation (Sears and Wong, 2001). Most important, since the enzymatic reactions are not optimized for high space-time yields (STY, g product L^{-1} h^{-1}), and the biocatalysts are not recovered and reused, biocatalyst recycling is economically demanding. Relevant numbers for STY and product-specific total turnover number TTN (g product/g catalyst) are therefore low for automated enzymatic synthesis with immobilized substrates and glycosyltransferases in solution.

2.2. Immobilized Leloir-glycosyltransferases

Automated glycan synthesis with immobilized glycosyltransferases offers several advantages including cascading, handling, and re-use of the biocatalysts as well as facile product isolation. This strategy was already used for the synthesis of different glycans (Fig. 7). However, only a few immobilized glycosyltransferases have been applied so far (Fig. 8, Table 3). Enzymes are immobilized onto a resin of solid support and trapped into different reaction columns. Free glycan acceptor, substrates, and other components are passed through the columns and the final product is recovered from the solution, analyzed, and prepared for another cycle of glycosidic elongation (Sears and Wong, 2001).

The immobilization of functional enzymes is not trivial. A lot of different parameters can affect the activity in a bound state. Key to high enzyme activity is the oriented immobilization mediated by terminal peptides/peptide tags or proteins. Conformation and orientation of immobilized proteins on the solid support can have a major influence on enzyme activity. A random orientation of enzymes could lead to a non-accessible active site. Therefore, careful experimental and construct design is essential (Cha et al., 2005). The selection of peptide tags for

Table 3
Enzymatic glycan synthesis with immobilized glycosyltransferases.

Glycosyltransferases	Products	Solid Phase	Tag	Process	References
• α-1,3-galactosyltransferase (bovine) • β-1,4-galactosyltransferase (bovine) • α-1,4-galactosyltransferase (*N. meningitidis*)	α-Gal Galili, Globotriose, and others Fig. 7, Structure 16	Ni^{2+}-agarose beads	His-tag	Repeated batch mode	(Nahalka et al., 2003)
• Glycosynthase β-*N*-acetyl-hexosaminidase D746E (*B. bifidum)*	lacto-*N*-triose II (LNT II)	Cu^{2+}-agarose	His-tag	Continuous flow synthesis	(Ruzic et al., 2020)
• β-1,4-galactosyltransferase (Human) • β-1,3-glucuronosyltransferase (Mouse)	human natural killer cell-1 (HNK-1) Fig. 7 Structure 20	Ni-IDA particles	His-tag	Automated flow reactor	(Heinzler et al., 2019)
• β-1,4-galactosyltransferase (Human)	LacNAc Fig. 7 Structure 17	Ni-IDA particles	His-tag	Automated flow microreactor	(Heinzler et al., 2018)
• *N*-acetylhexosamine-1-phosphate transferases Capsular polymerase CsaB, CsxA (*Neisseria meningitidis)*	Capsular polysaccharides CPSA and CPSX (Fig. 7 Structure 30)	HisTrap	His-tag	Continuous flow	(Fiebig et al., 2018)
• Hyaluronan synthase (PmHAS, *Pasteurella multocida*)	Hyaluronic acid (HA) Fig. 7 Structure 18	His Mag SepharoseTM Ni	His-tag	Repetitive one-pot reaction	(Gottschalk et al., 2022)
• β-1,4-Galactosyltransferase (Human, Toyobo Ltd.)	"LacNAc Polymer" Fig. 7 Structure 23	Glycolipid Membrane with Maltriose	Maltose binding Protein	Glycosyltransferase microarray chip	(Nagahori et al., 2003)
• β-galactoside α2,6-sialyltransferase (*Photobacterium* sp. JT-ISH-224)	Sialyllactose Fig. 7 Structure 25	Biotin-bearing magnetic microbeads	Fungal biotin-binding protein TM2	Single reaction	(Kajiwara et al., 2016)
• *N*-acetylglucosaminyltransferase I (GnTI) • α-Mannosidase II (ManII) • β-1,4-Galactosyltransferase (GalT)	Human-like glycosylated antibody fragments Free artificial glycans	Streptavidin silica beads	Biotin	Sequential glycosylation with enzyme recycling	(Makrydaki et al., 2022)
• C-glycosyltransferase (OsCGT, *Oryza sativa*) • Sucrose synthase (*Glycine* max; GmSuSy)	Nothofagin Fig. 7 Structure 29	ReliSorb SP400	$Z_{bascic2}$	Repetitive (semi) continuous synthesis	(Liu et al., 2021)
• CMP-sialic acid synthetase (CSS) (*N. meningitis*, NmCSS) • α-2,3-Sialyltransferase (*P. dagmatis*, PdSiaT).	α2,3-sialyl-β-D-galactoside Fig. 7 Structure 24	ReliSorb SP400	$Z_{bascic2}$	Cascade synthesis	(Schelch et al., 2022)
• α-1,4-fucosyltransferases (*H. pylori*) • α-1,3-fucosyltransferases (*H. pylori*) • β-1,4-galactosyltransferase (Human)	Lewis^x derivates Fig. 7 Structure 27	Membrane-mimetic magnetic bead	Terminal Cationic Amphipathic Peptide	Repeated synthesis	(Naruchi and Nishimura, 2011)
• OsEUGT11 (UGT1) • SrUGT76G1 (UGT2),	Rebaudioside M Fig. 7 Structure 26	Chitosan beads	None, glutaraldehyde cross-linking	One-pot synthesis	(Wang et al., 2021)
• β-1,4-galactosyltranseferase (Human) • α-1,3-fucosyltransferase (*H. pylori*)	Lewis^x Fig. 7 Structure 21	Sepharose	Linker anchoring Via Sortase A	Repetitive one-pot synthesis	(Ito et al., 2010)
• heparosan synthase 2 (PmHS2)	Heparan sulfate Fig. 7 Structure 19	Metal organic frameworks (MOFs)	None, encapsulation		(Qiao et al., 2022)
• α-1,2-galactosyl-transferase (GMA12+ from *S. pombe*) • α-1,2-mannosyltransferase (KRE2 from *S. cerevisiae*) • α-1,3-mannosyltransferase (MNN1 from *S. cerevisiae*)	O-linked oligosaccharides Fig. 7 Structure 22	Yeast Cell Surface	Pir protein		(Abe et al., 2003)
• 51 Human glycosyltransferases (Refer to publication for further information)	GM1b GD1, N-glycans, H antigen, Lewis^x, Lewis^y Fig. 7 Structure 28	Yeast Cell Surface	Pir protein		(Shimma et al., 2006)

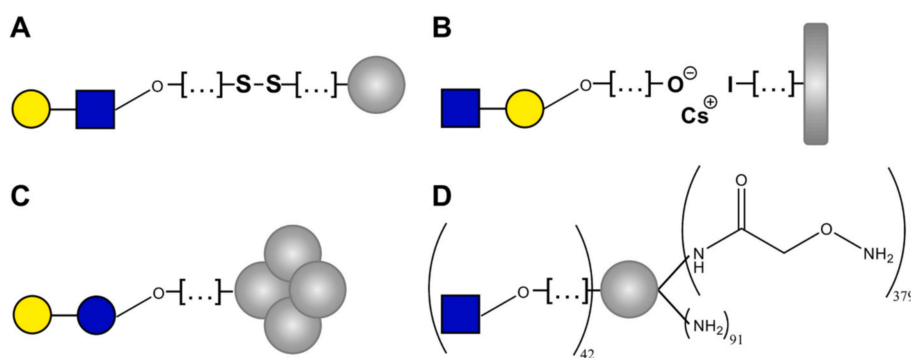


Fig. 5. Strategies for polymer-bound substrates. (A) Glycan acceptor is coupled with a thiol group and linked to thiopropyl Sepharose via disulfide formation (Blixt and Norberg, 1997). This strategy was also applied in a catch- and release approach using DTT to reduce the disulfide bond and isolate the product for a new cycle of glycosylation (Fang et al., 2022). (B) A halide linked to the solid support pore glass is nucleophilic displaced with the cesium salt of a carboxylic acid linked to the sugar molecule (Halcomb et al., 1994). (C) Glycans are immobilized to thermo-responsive soluble polymers. Increasing the temperature results in an insoluble phase (Huang et al., 2001; Zhang et al., 2018). (D) Poly(amidoamine) (PAMAM) dendrimer mimicking globular proteins which can be separated using ultrafiltration (Matsushita et al., 2010).

protein purification and/or later immobilization is essential. Different tags can severely affect enzyme stability, solubility, and activity (Kimple et al., 2013; Waugh, 2005). The solid phase can be chosen from a wide repertoire of different support materials, all with different advantages and features. Available materials differ in ease of handling, the potential of upscaling, stability, immobilization specificity or flexibility, or

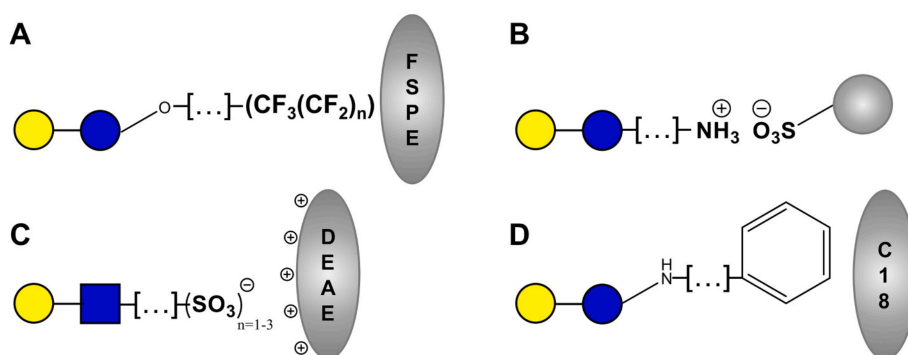


Fig. 6. Strategies for the transient catch and release of glycan products. (A) Acceptor sugar is linked to a fluorous tag ($(CF_3(CF_2)_n)$ and after glycosylation isolated using fluorous solid-phase extraction (FSPE) (Cai et al., 2014; Hwang et al., 2014; Jaipuri and Pohl, 2008). (B) Fusion of a small amino linker allows the formation of an ammonium cation under acidic conditions. The linked glycan was immobilized by a cation exchange resin (Zhu et al., 2017). (C) Acceptor sugars are equipped with a sulfonate moiety and captured on an anion exchange resin (Li et al., 2019a). (D) Hydrophobic carboxybenzyl (Cbz) protected glycans are captured by a C18 silica cartridge (Bai et al., 2022).

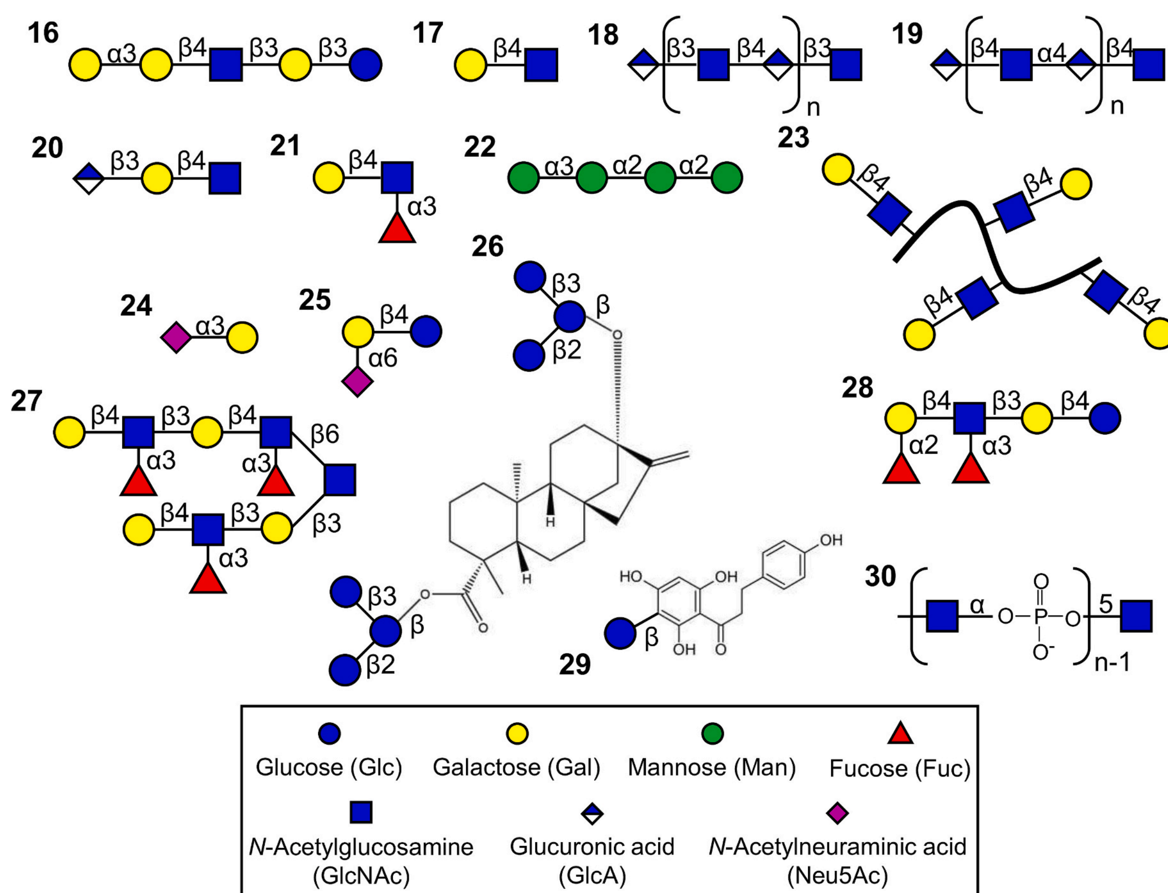


Fig. 7. Exemplary glycan structures of automated synthesis with immobilized GTs. **16** Globotriose, pentassaccharide with galili epitope (αGal) (Nahalka et al., 2003), **17** LacNAc type II (Heinzler et al., 2018), **18** hyaluronic acid (Gottschalk et al., 2022), **19** heparosan (Qiao et al., 2022), **20** non-sulfated HNK-1 epitope (Heinzler et al., 2019), **21** Lewis^x trisaccharide (Ito et al., 2010), **22** O-linked mannose oligosaccharide (Abe et al., 2003), **23** LacNAc type II polymer (Nagahori et al., 2003), **24** α2,3-sialyl-β-D-galactoside (Schelch et al., 2022), **25** sialyllactose (Kajiwara et al., 2016), **26** rebaudioside M (Wang et al., 2021), **27** branched HMO with Lewis^x antigen structures (Naruchi and Nishimura, 2011), **28** Lewis^y antigen (Shimma et al., 2006), **29** nothofagin (Liu et al., 2021), **30** Capsular polysaccharide X (Fiebig et al., 2018).

stiffness (Asanomi et al., 2011; Dohyun and Amy, 2013; Miyazaki and Maeda, 2006). Towards glycosyltransferase immobilization, several strategies have been described in recent years. While wide adaption of this technique is still lagging behind solid phase synthesis, the first success stories are published.

The Wang group showed efficient immobilization of different Leloir-type glycosyltransferases on so-called "super beads" (Nahalka et al., 2003) (Fig. 8A, Table 3). The enzymes have been immobilized via poly-histidine tags onto a Ni^{2+}-NTA resin and showed activities close to those in solution due to the directed orientation. Enzymatic glycosylation was performed with recycled enzymes. In addition, a sugar nucleotide synthesis system was included. Furthermore, two glycosyltransferases (β4GalT and α3GalT) were simultaneously immobilized on beads, offering the direct synthesis of complex glycan structures. A poly-histidine tag (His_6-tag) was also applied for the immobilization of a glycosynthase on metal-ion surface agarose by the Nidetzky group (Ruzic et al., 2020) (Fig. 8A, Table 3). The His_6-tag strategy was further exploited towards automation by using IMAC magnetic beads for glycan synthesis in a compartmented flow reactor, as described in chapter 4 (Heinzler et al., 2019; Heinzler et al., 2018) (Fig. 8A, Table 3). Furthermore, automated

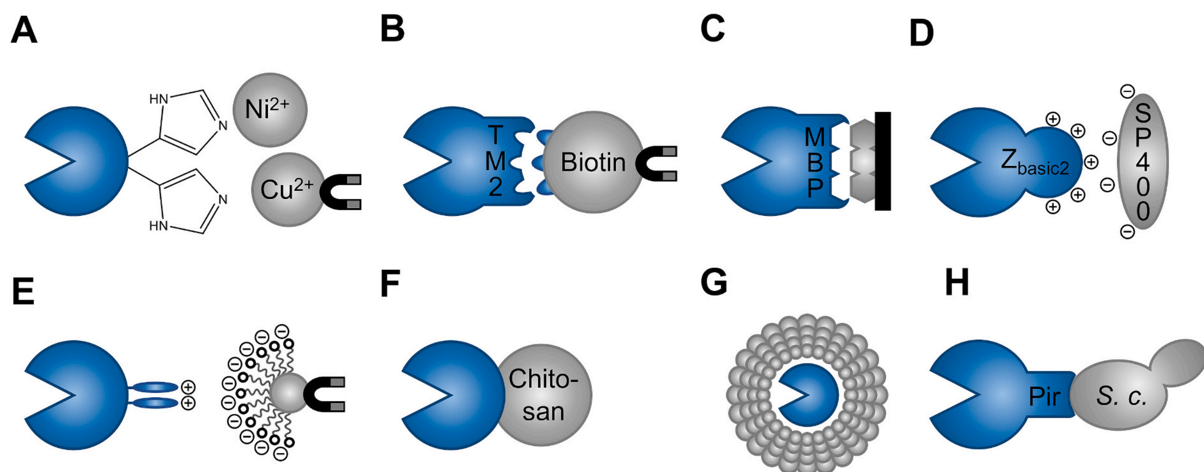


Fig. 8. Enzyme immobilization on a solid phase. (A) A poly-histidine tag allows ion metal affinity immobilization of glycosyltransferases on Cu^{2+} or Ni^{2+} beads, for certain strategies, magnetic beads are applied (Gottschalk et al., 2022; Heinzler et al., 2019; Heinzler et al., 2018; Nahalka et al., 2003; Ruzic et al., 2020). B) The fungal protein TM2 is capable to bind biotin. Glycosyltransferase-TM2 fusion proteins are immobilized onto biotin-bearing magnetic beads (Kajiwara et al., 2016). (C) A maltose binding protein (MBP) fused to the glycosyltransferase can interact with maltotriose on a solid phase for immobilization of the fusion protein (Nagahori et al., 2003). (D) Glycosyltransferases with the cationic binding module Z_{basic} are captured by a ReliSorb SP400 solid phase which offers sulfonate surface groups as negatively charged interaction sites (Liu et al., 2021; Schelch et al., 2020). (E) Glycosyltransferases often carry an N-terminal membrane anchor. Towards immobilization, two putative amphipathic α-helices fused to a glycosyltransferase allow membrane-bound glycosyltransferases on the membrane-mimetic magnetic bead (Naruchi and Nishimura, 2011). (F) Covalent attachment of glycosyltransferases to chitosan beads can be achieved by crosslinking reagents like glutaraldehyde or enzymatic by a transpeptidase reaction (Ito et al., 2010; Wang et al., 2021). (G) Encapsulation of glycosyltransferases in bimetal organic material allows immobilization of enzymes by just adding them to the polymeric reaction mixture (Qiao et al., 2022). (H) Cell surface protein Pir can be used to anchor glycosyltransferases on the surface of yeast cells (Abe et al., 2003; Shimma et al., 2006).

glycan synthesis on a HisTrap column was applied for synthesis of meningococcal capsular oligosaccharides using immobilized capsule polymerases (Table 1). Here, Gerardy-Schahn and colleagues were able to synthesize capsular oligosaccharides with a controlled length, the process is further described in chapter 4 (Fiebig et al., 2018). The Elling group also demonstrated the synthesis of hyaluronic acid by a cascade of seven immobilized enzymes in a one-pot reaction (Gottschalk et al., 2022) (Fig. 8A, Table 3). ATP regeneration and nucleotide sugar synthesis were also included. After each reaction cycle, the product solution was separated from the immobilized enzyme, retaining them for a new reaction cycle. To this end, higher activity of the immobilized enzyme was achieved with higher quality products (Gottschalk et al., 2022). However, His_6-tag immobilization is less stable than covalent coupling. Besides, the magnetic components significantly reduce the space available for enzyme immobilization and can lead to the inactivation of sensitive enzymes.

Yamamoto and co-workers described the immobilization of a sialyltransferase via a biotin-binding protein fusion to biotin-bearing magnetic beads (Kajiwara et al., 2016) (Fig. 8B, Table 3). Interestingly, while the initial activity of the immobilized glycosyltransferase was drastically lower in comparison to the soluble enzyme, the activity stayed constant over at least seven cycles. The stability of the immobilized sialyltransferase at higher temperatures and pH was increased in comparison to the free version (Kajiwara et al., 2016). Immobilization via biotin was also applied vice versa with biotinylated enzymes. In vivo biotinylation of an Avitag by the biotin ligase BirA was used for immobilization of glycosyltransferases on streptavidin coated solid supports (Makrydaki et al., 2022).

Another option was demonstrated by Nishimura and colleagues who applied a maltose binding protein (MBP) fusion for the immobilization of a β4-galactosyltransferase (Nagahori et al., 2003) (Fig. 8C, Table 3). Here, a polymer support having maltotriose branches was used for the immobilization of the enzyme by specific sugar interaction. The polymerized monolayer of a maltotriose-containing glycero-glycolipid can be transferred on a solid surface, for example, a glass plate or surface plasmon resonance sensor chip.

As an alternative to the unstructured His_6-tag, a Z_{basic2} module can be fused to the protein of interest (Gräslund et al., 2002; Liu et al., 2021). This small peptide forms a scaffold of charged amino acids and can bind to negatively charged surfaces like cation exchange resins or silica materials, driven by electrostatic interactions. Studies of the Nidetzky group demonstrate the use of the cationic Z_{basic2} module to immobilize two glycosyltransferases on ReliSorb SP400 particles (Liu et al., 2021) (Fig. 8D, Table 3). Although the activities of Z_{basic2}-tagged C-glycosyltransferase (OsCGT) and sucrose synthase (GmSuSy) were decreased in contrast to the corresponding streptavidin-tagged enzyme, the high recyclability of the immobilized enzyme allows extensive re-use strategies. Following this work, Z_{basic2} immobilization was recently applied for the immobilization of sialyltransferases as well as a CMP-sialic acid synthetase (Schelch et al., 2020) (Fig. 8D, Table 3).

A similar approach based on the charge of the solid phase support was established by Nishimura and co-workers (Naruchi and Nishimura, 2011) (Fig. 8E, Table 3). In their nature-inspired approach, they made use of the endogenous C-terminal region of *H. pylori* α1,3-fucosyltransferase (FucT) which is known to form two positively charged amphipathic α-helices that interact with negatively charged phospholipids in the plasma membrane. While it is described, that this region is associated with FucT activity it is also linked to low solubility and expression when produced in a heterologous host. For this reason, C-terminal truncated versions of the bacterial FucTs are often preferred (Heine et al., 2022b; Ma et al., 2003; Naruchi and Nishimura, 2011). However, this C-terminal tail was used for the design of a model peptide for specifically oriented enzyme immobilization. The model peptide forms the α-helical structure in the presence of *n*-dodecyl phosphocholine, and the conformational change allows the anchoring of glycosyltransferases to the surface of membrane-mimetic magnetic beads. The immobilized α1,3-FucT showed no significant loss of activity over 40 synthetic cycles in 10 days (Naruchi and Nishimura, 2011).

Co-immobilization of glycosyltransferases on chitosan beads offers the advantages of bead-immobilized enzymes with non-toxic and biocompatible properties of highly abundant and cheap chitosan. Song and co-workers co-immobilized two glycosyltransferases OsEUGT11 (UGT1) and SrUGT76G1 (UGT2) for glycosylation of Rebaudioside A and Rebaudioside D to Rebaudioside M. Here, conversion rates were

drastically increased in comparison to mixed immobilized enzymes (Wang et al., 2021) (Fig. 8F, Table 3). Although the activity of individual, immobilized enzymes was lower in comparison to free enzymes, recycling and reusability of this system made it economically advantageous already after two cycles (Wang et al., 2021). However, while the work described above used glutaraldehyde as the cross-linking reagent, conventional, chemical immobilization is associated with nonspecific, random, and multilocation cross-linking events, leading to a significant loss of enzyme activity. In an earlier work by Nishimura and colleagues, chemical activation of the solid supporting material was avoided by using enzymatic condensation of glycosyltransferases to Sepharose (Ito et al., 2010) (Fig. 8F, Table 3). *Staphylococcus aureus* sortase A (SrtA) was used to form a linkage between a motif at the C-terminus of the glycosyltransferase and the solid supporting material. In contrast to NHS or glutaraldehyde-based immobilization, the activity of SrtA-mediated immobilization retained almost all of its specific activity in comparison to the soluble enzyme. (Ito et al., 2010).

Encapsulation offers the advantage of stabilizing enzymes in a defined environment, shielding them from external pH and organic solvents. Huang and colleagues used zeolitic imidazolate (ZIF-90) Metal-organic framework (MOF) and divalent metal ions to generate these protective environments in bimetal organic material (BMOM) microreactors for encapsulation of *Pasteurella multocida* heparosan synthase 2 (PmHS2) (Qiao et al., 2022) (Fig. 8G, Table 3). In the end, the enzymatic activity of the encapsulated enzyme even exceeded the activity of the free enzyme. The enhanced activity was achieved by a microenvironment that mimics the milieu in living cells, protecting from an inhospitable external environment and a steric orientation that favors enzymatic reaction (Qiao et al., 2022).

Besides the described solid phase support for enzyme immobilization, rather unconventional immobilization techniques offer the potential to produce products in niche applications. Anchoring of glycosyltransferases to the surface of *Saccharomyces cerevisiae* cells is one alternative to classic immobilization (Abe et al., 2003) (Fig. 8H, Table 3). The group of Jigami showed the exposure of glycosyltransferases on the cell surface was realized by using the yeast cell protein Pir. Cells expressed and secreted glycosyltransferases-Pir fusion proteins that covalently bind to cell wall components. The combination of enzyme production and subsequent display on the production host cell wall allowed the synthesis of glycan structures in a culture medium (Abe et al., 2003). This technique was further improved by the same group for the immobilization of 51 human glycosyltransferases on yeast cell surface (Shimma et al., 2006). To this end, 40 glycosyltransferases showed activity on the cell surface and were applied for the synthesis of different glycans such as Lewis^x, Lewis^y, or H-antigen.

2.3. Protein immobilization beyond glycosyltransferases

The repertoire of protein immobilization techniques was applied to manifold more strategies for enzymatic reactions besides glycosylation on solid phase support. Protein fusions such as Halo-Tag (33 kDa) (England et al., 2015) or SNAP-Tag (19.4 kDa) (Juillerat et al., 2005) react with chloro-functionalized ligand or benzyl-guanine, respectively. Enzyme immobilization in aqueous microgels has been developed offering several advantages (Nöth et al., 2020; Zou et al., 2019). The 3D colloidal polymer networks of aqueous microgels provide an ideal bio-inspired environment for the enzymes and maintenance of their activity and long-term stability. The porous microgel structure is advantageous for the diffusion of reagents to the active sites of enzymes and the release of the product. The stimuli-responsiveness of microgels allows for regulating their swelling degree, polarity, and diffusion processes in their interior by temperature, pH, or light. This offers a unique possibility to adjust the internal structure of microgels to an optimal catalysis reaction temperature of enzymes (30 °C) and trigger enzymatic reactions in microgels (on/off function). One promising approach was the immobilization of enzymes into microgels by the crosslinking of reactive pre-polymers in the presence of the enzyme. Reactive copolymers based on methacrylic acid *N*-hydroxysuccinimide ester (MNHS) (Peng et al., 2015); glycidyl methacrylate (GMA) (Peng et al., 2016b) or pyridyl disulfide ethyl methacrylate (PDSM) (Peng et al., 2016a), respectively, and cyclic *N*-vinylamides such as *N*-vinylpyrrolidone (VP), *N*-vinylpiperidone (VPI) or *N*-vinylcaprolactam (VCL) were synthesized by RAFT polymerization. Microgel-encapsulated cellulase showed improved stability towards enhanced temperature, chaotropic agents, and organic solvents and positive effects on the storage ability (Peng et al., 2019; Peng et al., 2016b). Cellulase could be released from the PDMS functionalized microgels by the addition of dithiothreitol (DTT) for cleavage of disulfide crosslinks (Peng et al., 2016a). In a recent study, the synthesis of amylose-coated, PVCL microgels by enzyme-catalyzed grafting-from polymerization was demonstrated (Gau et al., 2019). The phosphorylase can recognize the oligosaccharide maltoheptaose attached as a primer to the PVCL microgel chains and attach glucose units from the monomer glucose-1-phosphate forming a thick amylose-based shell.

While different techniques describe a transient immobilization of enzymes to the solid phase, others use a covalent binding. One example of an irreversible attachment of enzymes to a solid support is the exploitation of the SpyCatcher/SpyTag system, originating from a *Streptococcus pyogenes* protein. Here, both peptide tags can be genetically fused to a protein of interest and autocatalytically form a covalent isopeptide bond (Reddington and Howarth, 2015). The SpyCatcher/SpyTag system appears as highly attractive because of the small sizes of the fusion fragment (SpyCatcher with 12.5 kDa) and amino acid tag (SpyTag with 13 aa) (Keeble et al., 2017; Reddington and Howarth, 2015; Sutherland et al., 2019). An elegant application of this system was described recently by Mittmann and colleagues who attached SpyTag-tagged decarboxylase onto SpyCatchers-magnetosomes (Mittmann et al., 2022). Decarboxylase-SpyTag immobilized on SpyCatcher-magnetosomes was used for continuous flow biocatalysis. Therefore, the magnetic nanoparticle catalysts were loaded into a reactor and fixed via magnets in a continuous reaction (Mittmann et al., 2022). Further applications of the SpyCatcher/SpyTag system are wide-reaching and could include hydrogel formation and immobilization, cyclization of enzymes, or generation of larger enzymes complex for "molecular conveyors" (Reddington and Howarth, 2015; Zhong et al., 2022). Self-assembling of SpyCatcher/SpyTag modified alcohol dehydrogenase and glucose-6-phosphate dehydrogenase formed enzyme hydrogels for the synthesis of diols with cofactor regeneration in a flow microfluidic reactor (Peschke et al., 2018).

Recent studies applied the SpyCatcher/SpyTag technology for sugar synthesis or conversion. SpyTag-glycosidases were assembled in a polymeric SpyCatcher scaffold and used as hemicellulosome (Jia et al., 2017). Substrate conversion was enhanced compared to the free enzymes and attributed to the close spatial proximity. Ni and co-workers designed a cyclized β-Galactosidase BgaB for the synthesis of galactooligosaccharides (Han et al., 2022). The cyclized enzyme showed increased stability while conformation or secondary structure was not significantly altered. In another study, Qin and colleagues used the SpyCatcher/SpyTag interaction for the immobilization of D-allulose 3-epimerase (DAEase) to a solid resin (Gao et al., 2023). Conversion of D-fructose into D-allulose was achieved in multiple cycles with the immobilized enzyme over a prolonged time with only very little loss in activity. However, we have shown the first example of a SpyCatcher Leloir glycosyltransferase fusion protein suitable for enzymatic glycan synthesis (Palm et al., 2022).

3. In vitro enzyme synthesis and use for glycan synthesis

Other interesting and promising approaches for versatile glycan synthesis and an extension of the "Glyco-Toolbox" are "cell-free" processes. It has to be stated that nearly all common synthesis processes for glycan structures are performed with purified GTs and therefore are cell-

free. However, the term "cell-free glycan synthesis" characterizes approaches for in vivo, in situ, or in vitro expression of GTs for in vivo, in situ, or in vitro protein glycosylation or glycan synthesis, and multi-step syntheses that combine these methods. The DeLisa group has pioneered this topic on cell-free glycan/glycoprotein synthesis. At this point, we refer to their very detailed review regarding this topic (Jaroentomeechai et al., 2020) of which we will summarize the most important findings in connection to automated enzymatic glycan synthesis.

The term "cell-free biology" was first introduced about 60 years ago in connection to deciphering the genetic code (Nirenberg and Matthaei, 1961). Today it is used to describe the production of complex biomolecules, optimization of metabolic pathways, and building of genetic networks. Methods for the specific synthesis of glycans and derived structures are nowadays summarized under the term "synthetic glycobiology". It was first introduced to describe the redesign of GT assembly lines and design via protein engineering (Czlapinski and Bertozzi, 2006). In pioneer studies, the N-linked glycosylation machinery from *Campylobacter jejuni* was transferred into *E. coli* strains and herewith giving these the ability of protein glycosylation (Wacker et al., 2002). While usually until today living organisms are involved in synthetic glycobiology, recently cell-free systems gained higher popularity. Their advantages are better possibilities for examination, higher flexibility, and more stringent control over glycan assembly. Moreover, in these systems, protein expression and glycan synthesis are independent of cell viability (Swartz, 2018). As mentioned above, mostly cell-free synthetic glycobiology involves purified enzymes that catalyze specific glycosylation reactions in vitro. However, in situ production of GTs and other glycosylation enzymes coupled with multi-step glycosylation reactions in cell-free lysates is also possible and is being researched intensively (Jaroentomeechai et al., 2018).

In general, synthetic glycobiology can be divided into several individual research fields. In the following, three of the most interesting ones are listed and important research results regarding these fields are explained:

Cell-free enzymatic synthesis of nucleotide sugars. Since the synthesis of nucleotide sugars on a multi-gram scale has become an area with a major focus on cell-free synthesis (Frohnmeyer and Elling, 2023), it is now possible to build UDP-Glc, UDP-Gal, UDP-GalNAc UDP-GlcNAc (Fischöder et al., 2019; Schmölzer et al., 2017), UDP-GlcA (Gottschalk et al., 2021; Muthana et al., 2015), UDP-Xyl (Eixelsberger and Nidetzky, 2014; Shi et al., 2022; Wang et al., 2018), GDP-Man (Wang et al., 2022), and GDP-Fuc (Frohnmeyer et al., 2022) and therefore all common nucleotide sugars.

Enzyme-mediated assembly of glycans and glycolipids. The GTs OleD from *Streptomyces antibiotics* and YijC from *Bacillus licheniformis* catalyze the formation of O-, N-, and S-glycosidic linkages (Dai et al., 2017; Gantt et al., 2008). This opens up possibilities for in vitro glycosylation of small molecules like flavonoids, alkaloids, and antibiotics. Furthermore, libraries for human and bacterial glycolipids have been created using cell-free synthesis (Glover et al., 2005; Li et al., 2019b).

Cell-free biosynthesis of glycoproteins. The ability to operate glycoprotein biosynthesis in a well-defined environment allows for obtaining various well-defined glycoproteins with high purity and could rapidly enhance the glycoscience regarding therapeutic proteins and vaccines. While earlier studies focused on glycoprotein synthesis by chemical or chemoselective ligation, nowadays different routes seem to be more promising. For example, endoglycosynthase-mediated chemoenzymatic glycan remodeling has become an efficient method to generate homogeneous glycoforms of therapeutic mAbs with relatively large glycoform libraries (Lin et al., 2015). Moreover, the en-bloc transfer of glycans to proteins mediated by oligosaccharyl transferases (OSTs) is also possible in cell-free systems. Here, a glycan precursor is transferred to an asparagine residue in an Asn-Xaa-Thr/Ser motif for *N*-glycosylation and then undergoes several trimming steps by glycosidases and/or building steps by GTs (Aebi, 2013). Furthermore, it was discovered, that certain *Neisseria* and *Pseudomonas* species express enzymes that enable O-linked en-bloc glycan transfer (Faridmoayer et al., 2007). With that, many options for novel glycoproteins unfold. The DeLisa group has done several studies on GT-mediated protein glycosylation and glycan elaboration. Especially their GlycoPRIME (glycosylation pathway assembly by rapid in vitro mixing and expression) technology has to be highlighted. With that, they established a cell-free expression route for 24 bacterial and eukaryotic GTs in *E. coli* and utilized these to generate 18 unique glycan structures, including α1–3-linked galactose (αGal) epitope as well as fucosylated and sialylated lactose or poly-*N*-acetyllactosamine (LacNAc) which were built on a single glucose residue that was installed on a model protein via *Actinobacillus pleuropneumoniae* *N*-linked glycosyltransferase. Moreover, they established a one-pot synthesis (cell-free protein synthesis driven glycoprotein synthesis, CFPS-GpS) for selective glycosylation. In this system, the target protein is co-expressed with GTs in the presence of sugar donors to simultaneously synthesize and glycosylate the glycoprotein of interest in an *E. coli* lysate. By doing so, the researchers have proven that it is possible to utilize cell-free in situ expression and glycosylation platforms in a one-pot reaction for the fast and selective production of therapeutic glycoproteins (Kightlinger et al., 2019).

As stated previously, it is highly desirable to integrate glycan synthesis into automated processes. The same applies to cell-free syntheses. Since the biological membrane boundary is nonexistent in cell-free systems, it is thought, that this opens the integration of high throughput screening tools and real-time monitoring of said processes (Georgi et al., 2016; Zhang et al., 2019). With that, especially the time-consuming initial assessments of novel processes can be shortened.

By further developing the stated processes and integrating novel analytical tools and techniques, the automated synthesis of glycans, small glycosylated molecules, and selective glycoproteins seems to be within a reachable distance.

4. Reactor types for enzymatic glycan synthesis

Continuous flow biocatalysis in bioreactors has rapidly developed over the past years (Britton et al., 2018). Here, a liquid phase containing the starting substrates is pumped continuously through the reactor, where they are converted, to yield the desired products after the reaction. Enzymatic reactions occur at the stationary phase, containing the immobilized biocatalysts, through interaction with the mobile/liquid phase (Liu et al., 2016). Biocatalysis in a flow reactor is often associated with increased efficiency and conversion rate, thus being more feasible than batch production (Britton et al., 2018).

In the following, the four main strategies for flow reactors with immobilized enzymes are described, namely **(i)** open capillary enzyme reactors; **(ii)** monolithic enzyme reactors; **(iii)** fixed-bed enzyme reactors, and **(iv)** enzyme membrane reactors (EMR) (Liu et al., 2016). Excellent further reading including immobilization strategies in the reactor, examples of products and their analysis, and comparison of reactor systems are presented in recent reviews (Liu et al., 2016; Meller et al., 2017). Furthermore, immobilization strategies are described in Chapter 2 of this review.

In **(i)** open capillary enzyme reactors, the inner wall of capillaries or microfluidic structures is designed for immobilization of the biocatalysts to a support layer (Liu et al., 2016). However, with the increasing diameter of the capillaries or channel structures, the surface-to-volume ratio of this type of reactor quickly decreases, resulting in low conversion rates and low space-time yields (STY) (Meller et al., 2017).

The reason behind this phenomenon can be attributed to the decreased efficiency of multiple enzymes that catalyze sequential step of a reaction. While employing a multi-enzyme coated microchannel yields a greater product yield compared to a multi-enzyme bead packed bed method, the reduced efficiency in substrate conversion requires a manifold higher utilization of expensive individual enzymes. Particularly when combined with high flow rates, a large portion of the substrate remains unconverted, leading to a diminished substrate

conversion efficiency per enzyme (Boehm et al., 2013). This results in a lower substrate conversion efficiency per enzyme (Boehm et al., 2013; Liu et al., 2016).

Better use of the reactor volume is reached for **(ii)** enzymes directly immobilized onto monolithic structures in the reactor. Here, mass transport mainly takes place via convection, offering higher accessibility of the active site of the biocatalyst for substrates as well as higher stability and versatility of functional groups (Liu et al., 2016; Meller et al., 2017). Still, their production is relatively complex and scalability is limited. Besides, the renewal of the immobilized enzyme either requires disassembling the reactor and replacement of the complete monolithic structure or elaborate detachment and re-coupling chemistries.

Therefore, (**iii**) fixed-bed enzyme reactors are often chosen because they offer a high surface-to-volume ratio of the used porous enzyme carriers, use the full reactor volume for the enzymatic reaction, and are easily scalable (Boehm et al., 2013; Liu et al., 2016). For fixed-bed enzyme reactors, different micro- or nanoparticles and a variety of immobilization methods, such as covalent coupling, entrapment, or cross-linked enzyme aggregates are used. However, independent of the used carrier structure, fixed bed enzyme reactors normally use simple designs not offering additional functionalities, such as in-situ product removal.

In contrast, (**iv**) enzyme membrane reactors (EMR) can operate with free and/or immobilized enzymes. Excellent overviews of available reactor types and existing applications are provided by reviews from Calabrò (Calabrò, 2013) and Giorno et al. (Giorno and Drioli, 2000). Oligosaccharide synthesis in EMR was discussed by Su and colleagues (Su et al., 2020).

In the first type of EMR, the biocatalyst is active in a homogeneous solution and continuously fed with the substrate solution. After the conversion of the substrate, the enzyme is retained by an ultrafiltration membrane while the product can pass this membrane and is therefore removed from the reaction (Calabrò, 2013). However, if operated as dead-end filtration the enzyme quickly accumulates above the membrane surface leading to concentration polarization and potential fouling. Cross-flow operation of the EMRs and recycling the reaction solution in a loop reduces concentration polarization, however, the concept is only partly suitable for the realization of continuous flow enzymatic cascades, especially if the involved substrates and products are of comparable size. Taking these points into account, higher enzyme vulnerability and loss of activity are considerable cost factors due to the need for recovery and new supplements of active biocatalysts. Besides, the corresponding depletion of the enzyme in the bulk volume of the reactor results in decreased conversion rates and STY.

In the second type of EMR, the enzyme is immobilized within the membrane matrix itself, avoiding extensive shear stress and resulting activity loss to biocatalysts (Calabrò, 2013; Giorno and Drioli, 2000; Su et al., 2020). Furthermore, the stability of enzymes can be improved when they are immobilized on the solid support in comparison to enzymes in solution. However, in this case, the contact time between the substrate passing the membrane and the immobilized enzyme is very short for continuous single-pass systems, reducing the achievable throughputs and yields. Therefore, most scientific and industrial reports of EMR applications use systems of the first type with free enzymes (Hilterhaus and Liese, 2009). Mostly a combination of continuously stirred tank reactors (CSTR) and commercial membrane filtration units are applied, however, such a combination is far from an optimized, fully integrated EMR unit suitable for complex enzymatic cascades.

4.1. Application of reactors for glycan synthesis

However, while continuous flow bioreactors have been used for numerous biocatalytic reactions (Britton et al., 2018; Giorno and Drioli, 2000), only a few studies describe the application for automated glycan synthesis. Nevertheless, some promising examples demonstrate the potential of different reactor systems for automated glycan synthesis (Fig. 9).

4.1.1. Peptide synthesizer

The reactor system is based on a commercial peptide synthesizer (CEM Liberty Blue peptide synthesizer) and a thermoresponsive polymer for substrate immobilization (Zhang et al., 2018) **(**Fig. 9A). At a reaction temperature below the lower solution temperature (LCST), poly(*N*-isopropylacrylamide) (PNIPAM) is in a soluble state. Rising the temperature renders PNIPAM insoluble. In the current application, lactose with an aminopropyl linker was first chemically conjugated to PNIPAM and served as a primer substrate (**chapter 2.1.1.** and Fig. 5C). Soluble glycosyltransferases, nucleotide sugar building blocks, and reaction buffers are stored in preexisting tubes. In multiple cycles, the compounds are automatically injected into the reaction vessel to start the reaction. With the start of a cycle, glycosylation is performed on the soluble polymer-bound glycan at 25 °C. After the enzymatic elongation of the polymer-bound glycan, the reaction vessel is heated to 90 °C, resulting in precipitation of the polymer. Remaining buffer compounds, denatured enzymes, nucleotides, and nucleotide sugars are removed by washing steps while the precipitated polymer with glycans is retained by filtration. The soluble GT is denatured at the heating step and discarded. For the next cycle, the temperature is lowered to 25 °C to re-solubilize the polymer, and new sugar nucleotide, GT, and fresh buffer are added. After the final glycosylation step, the product is chemically cleaved off the polymer and purified via HPLC. As an example, GM1 ganglioside (**product 4** in Fig. 4**)** synthesis was performed with three glycosylation cycles starting from lactose: 3 h for NeuAc transfer, 6 h for GalNAc transfer, 6 h for Gal transfer utilizing enzymes PmsT1, CgtA, and CgtB, respectively, resulting in a total process time of 15 h with 20 mg product in 38% yield (Zhang et al., 2018).

4.1.2. Automated dispenser apparatus

The Boons group utilized an automated dispenser apparatus (ChemSpeed ISYNTH AI SWING platform) in combination with a catch and release strategy for intermediate product recovery (Li et al., 2019a). All liquid handling steps are programmed and performed by the dispenser in an automated sequence. In this application, a sulfonated acceptor glycan is mixed with the reaction buffer, containing the sugar nucleotides, GT, and all buffer components in a reaction vessel. After incubation and glycosylation, the reaction solution was transferred to a solid phase extraction rack, containing a diethyl aminoethyl (DEAE) anion exchange resin to catch the sulfonate-tagged glycan (Fig. 6C) and remove remnant buffer compounds, nucleotide sugar, and enzyme. After elution by ammonium bicarbonate and adjustment of pH, the sulfonate-tagged glycan is transferred to the next reaction vessel for a new glycosylation cycle (Fig. 9B). In the final step after HPLC purification, the sulfonate tag is cleaved off the glycan product by mild acidic conditions. As an example, starting from a sulfonate-tagged lactose a branched HMO (**product 11** in Fig. 4) was obtained in six reaction cycles with an 85% average yield per step after approximately five days (Li et al., 2019a).

4.1.3. Immobilized enzymes in a compartmented flow reactor

For automating enzyme cascade reactions, immobilized microfluidic enzyme reactors (IMER) are of special interest (Heinzler et al., 2019; Heinzler et al., 2018). In an IMER, paralleled immobilized enzyme modules process substrates, and intermediate products are transferred for conversion in subsequent enzyme modules. Purification of intermediate products is not required thanks to optimized substrate conversion in the spatially separated enzyme modules, which reduces processing time and boosts space-time yields. By combining compatible enzymatic reactions in various configurations under optimal conditions and offering the ability of cost-benefit analysis before scale-up, the IMER makes it possible to construct effective enzyme cascades. These characteristics are particularly valuable for automating enzymatic glycan production. Magnetic enzyme beads (MEBs) were prepared by immobilization of

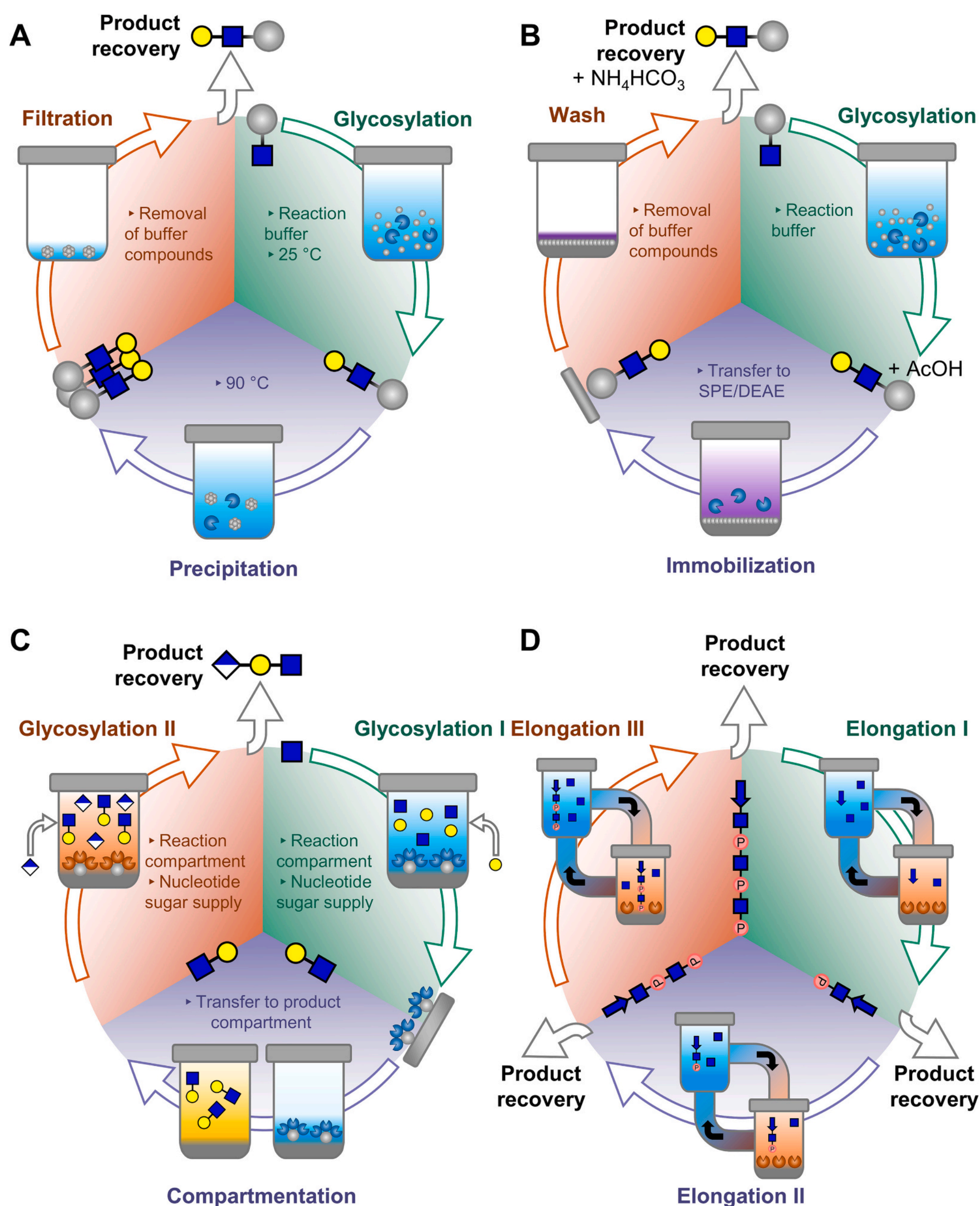
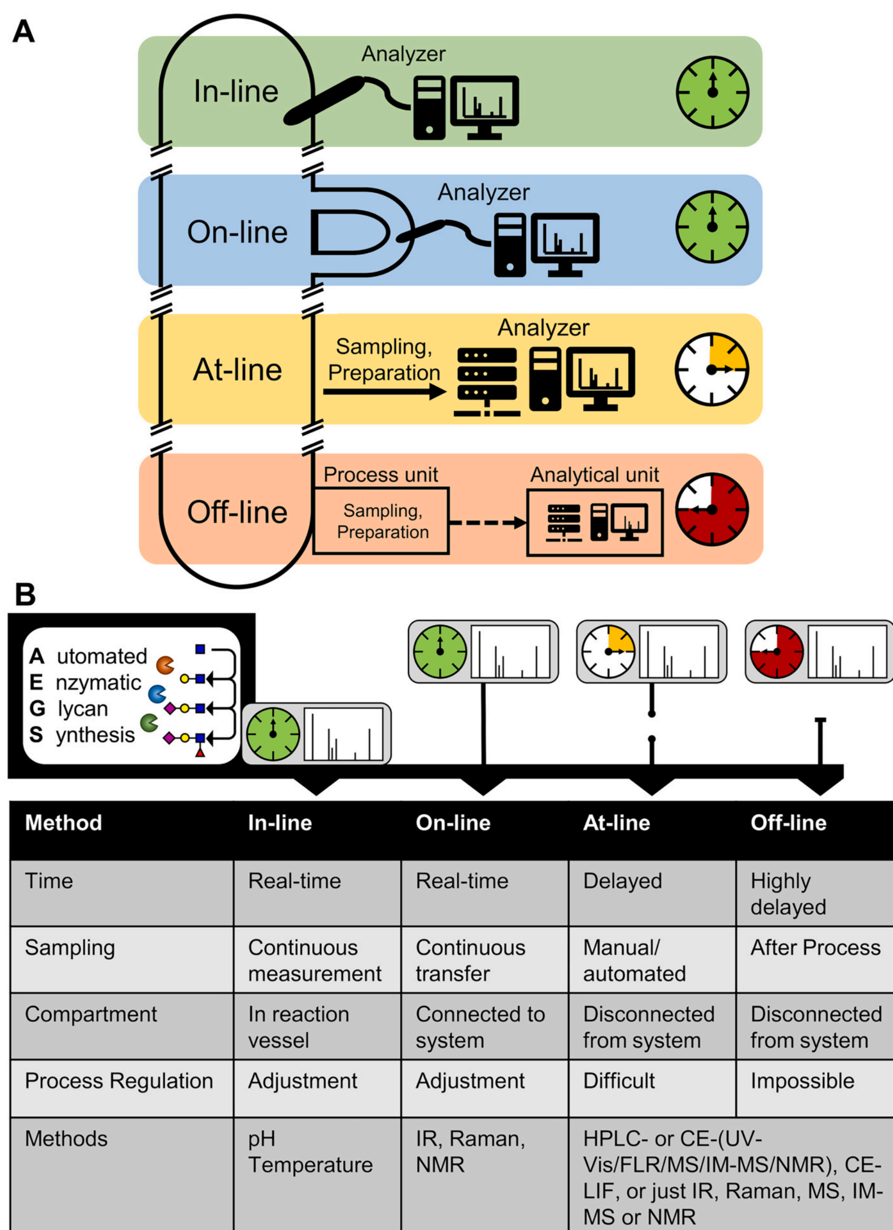


Fig. 9. Automated oligosaccharide synthesis in bioreactors. (A) Peptide synthesizer-based synthesis by Wang and colleagues (Zhang et al., 2018). Green Area: Polymer-bound (grey) glycan acceptor GlcNAc is embedded in the reaction vessel. Glycosyltransferase (blue), nucleotide sugar, and reaction buffer is added. Enzymatic glycosylation is performed at 25 °C to ensure the soluble state of the thermo-responsive polymer. Purple Area: Increasing the temperature to 90 °C results in precipitation of the thermo-responsive polymers. Orange Area: The liquid phase is removed by filtration, leaving only the polymer-bound glycans in the reaction vessel, the final product LacNAc is recovered. (B) automated platform liquid dispenser for glycan synthesis using capture and release of glycan products by Boons and co-workers (Li et al., 2019a). Green Area: The sugar acceptor GlcNAc is linked to a sulfonated tag (grey). In a reaction vessel (blue column), nucleotide sugar, glycosyltransferase (blue), and buffer reagents are mixed for the glycosylation of the acceptor sugar. After glycosylation, acidification (+ AcOH) and transfer of the reaction mixture to a DEAE-SPE column (purple area) allow the capture of the sulfonate-tagged glycan. Orange Area: The resin is washed to remove enzymes, nucleotide sugars, and other components. For product recovery, the pH is increased (+ NH_4HCO_3). (C) Automated glycan synthesis in an immobilized microfluidic enzyme reactor (IMER) by Franzreb and colleagues (Heinzler et al., 2019). Enzyme modules harboring enzymes for nucleotide sugar synthesis (not depicted) and glycosyltransferases, all immobilized on magnetic beads (MEBs), are compartmented within the reactor. Green area: Reaction vessel containing MEBs for glycosylation. Purple area: After the enzymatic reaction, the reaction mixture is transferred to a product compartment, where MEBs are held back by a magnetic field. Orange Area: Transfer of the glycan intermediate from the product compartment to a new reaction compartment, harboring another glycosyltransferase and supplemented with the respective buffer and nucleotide sugar. The nucleotide sugars are produced in parallel in distinct reaction compartments (not shown) and transferred to the glycosylation reaction compartment. (D) Automated solid-phase production of capsular meningococcal homopolymer oligosaccharides (Fiebig et al., 2018). A reaction mix containing UDP-GlcNAc and capsular polysaccharide acceptor with a degree of polymerization of five (primCPSX, blue arrow) is continuously circulated from the reservoir (blue column) to a reaction column containing immobilized recombinant capsular polymerase (CsxA) (orange column). A second immobilization column downstream of the reaction column prevents the enzymes from bleeding to the reservoir (not shown). Separation of CsxA from the product after each elongation step (green, purple, orange area) and circulation of the new product through the system allows the generation of uniform oligosaccharides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

His$_6$-tagged enzymes on Ni^{2+}-IDA particles (Fig. 8A). Enzyme modules consisted of MEBs for the synthesis of UDP-Gal and UDP-GlcA, respectively, and two GTs (β4GalT and β3GlcAT). The resulting four EMs were cascaded in an automated compartmented flow microreactor system (CFMS) for the synthesis of the non-sulfate human killer cell (HNK-1) epitope (Fig. 9C, **product 20** in Fig. 7). Compartmentation was achieved by magnetic fixation of MEBs in the reactor system. The nucleotide sugars were synthesized in parallel by the compartmented EMs and transported by flow to the respective glycosylation compartment. Starting from a linker-modified monosaccharide (GlcNAc-tBoc), the intermediate product LacNAc-tBoc was first synthesized and transported to the next compartment. The tri-saccharide product was obtained after 210 min overall reaction time in 96% yield (Heinzler et al., 2019).

4.1.4. Immobilized glycosyltransferases in plug-flow column reactors

The production of two capsular meningococcal oligosaccharides was achieved by solid phase fixation of different versions of glycosyltransferases and a controlled donor-to-acceptor ratio for the formation of homopolymers (CPSX: [α4GlcNAc-1-P]$_{n-1}$GlcNAc; CPSA: [α6ManNAc-1-P]$_{n-1}$ManNAc) with a defined size (Fiebig et al., 2018) (Fig. 9D, **product 30** in Fig. 7). Here, a hexose-1-phosphate transferase was engineered to a distributive mode of action, resulting in dissociation after each transfer of new donor substrate and therefore synthesis of uniform oligosaccharides. Consequently, the degree of polymerization of the homopolymer can be controlled by the ratio of the priming acceptor to donor sugar. Exemplary, a 1:20 ratio containing the capsular polysaccharide acceptor (primCPSX) and UDP-GlcNAc, respectively, was continuously pumped through the system, passing through the reaction column, containing immobilized capsular polymerase (CsxA) enzyme. Parallel HPLC monitoring of the reaction revealed complete consumption of the sugar nucleotide after 100 min. Analysis of the product showed a controlled polymerization, comparable to conventional production protocols. Furthermore, the generation of the sugar donor UDP-ManNAc from the more stable precursor UDP-GlcNAc by a 2-



Method	In-line	On-line	At-line	Off-line
Time	Real-time	Real-time	Delayed	Highly delayed
Sampling	Continuous measurement	Continuous transfer	Manual/ automated	After Process
Compartment	In reaction vessel	Connected to system	Disconnected from system	Disconnected from system
Process Regulation	Adjustment	Adjustment	Difficult	Impossible
Methods	pH Temperature	IR, Raman, NMR	HPLC- or CE-(UV-Vis/FLR/MS/IM-MS/NMR), CE-LIF, or just IR, Raman, MS, IM-MS or NMR	

Fig. 10. Comparison of process analytical technologies (PATs). (A) Schematic visualization of spatiotemporal differences in in-line, on-line, at-line, and off-line analyses. (B) Tabularization of differences between different PATs.

epimerase was established, either parallel in-process or by fusion of the glycosyltransferase with the epimerase. The acceptor and donor substrates were continuously pumped through the column and provided CPSX oligosaccharides with low polymer dispersity (Fiebig et al., 2018). A similar protocol was applied for the synthesis of capsular oligosaccharides from *Actinobacillus pleuropneumoniae* serotype 1 (Budde et al., 2020). In summary, the described reactor system with immobilized GTs is highly suitable for the efficient synthesis of vaccine oligosaccharides (Oldrini et al., 2018).

5. Product analysis and process monitoring in AEGS

Simultaneously with the increase of glycan synthesis processes, the need for fast and precise analysis methods of glycans came into focus. Since the field of applications grew rapidly, the term *omics* was introduced to glycan analytics as it was done with protein and genome analytics (*proteomics* and *genomics*). The term *glycomics* comprises the various technologies that enable or help to decipher glycans which were taken from biological samples or synthesized with methods previously described (Bertozzi and Sasisekharan, 2009).

In general, the analytical monitoring of a product is a cost factor that should not be underestimated. Since glycans are not only defined by their monosaccharide composition, but also by the linkage type of each glycosidic bond, anomeric configuration, the branching, and conformation of each monosaccharide, a reliable and precise analytical workflow is of high interest.

In processes such as (chemo-)enzymatic glycan assembly or protein glycosylation, not only the product formation but also the reaction parameters have to be monitored. For this, several tools, devices, and monitoring software are available. In general, the needed equipment is summarized by the term process analytical technology (PAT). PATs are divided into four classes: in-line, on-line, at-line, and off-line (Fig. 10A). In dependency on the analytical objective and its feasibility, PAT tools are differently applied to the process (Minnich et al., 2016; Morin et al., 2021):

In-line: Analytical probes for in-line measurements are directly inserted into the reaction vessel. Regarding AEGS, well-known probes are temperature or pH electrodes that are applied to a bioreactor or flow cell (Fig. 10B). Parameters measured in-line can be monitored and evaluated in real-time and adjusted while the process is still running. In-line glycan product analysis appears as difficult to install in the synthesis process.

On-line: In on-line measurements, the analyte is bypassed to a connected analyzer. Similarly, to the in-line analysis, the measurement is in real-time and in direct connection to the reaction vessel. The diverted stream is returned to the reaction vessel post-analysis. On-line PATs are advantageous when adjustments on the analyte are needed for a precise analysis while the reaction processes and its parameters must be stable. For example, the pressure, temperature, and flow velocity can be modified without altering the process parameters of the main reaction. On the negative side, the equipment effort and costs are higher than for in-line PATs. Suitable analytical methods, but not yet applied for on-line product glycan product analysis, are e.g., IR and Raman spectroscopy, or NMR (Fig. 10B).

At-line: The analyzer for at-line analytics is disconnected from the process. Therefore, manual or automated sampling is required. In contrast to the PATs mentioned before, this opens the opportunity to further prepare the analyte for several analytical applications. Although analytical technologies are advancing in sample preparation and analysis time, the monitoring of product formation and adjustment of process parameters are delayed. However, for product analysis, at-line PATs are most suitable, utilizing highly precise analytical methods such as HPLC-(UV-Vis/FLR/IM-MS/NMR, CE-(UV-Vis, FLR/LIF/MS/IM-MS), or (for not too complex processes) just IR, Raman, IM-MS, or NMR (Fig. 10B).

Off-line: For off-line analysis, a sample is taken from the process, as it is done for at-line analytics. However, sample preparation and product analysis are completely uncoupled from the process. The sample is delivered to a separate analytical unit and the time interval between product formation and product analysis is the largest of these four PATs.

Since the analysis of glycan structures often requires a laborious and time-consuming sample preparation, in-line and on-line glycomics are hardly possible and not feasible to this day. However, over the last years, several analytical methods for glycans were developed and further improved. A very detailed outline of glycan analytics can be found in a recent review by Rapp and colleagues (Pralow et al., 2021). Here, we summarize the most important conclusions on the state-of-the-art glycan analysis and asses them regarding AEGS:

Well-known established technologies for glycan analytics are categorized into three groups: Liquid chromatography (LC)-based, mass spectrometry (MS)-based, and capillary electrophoresis (CE)-based glycan analytics. All methods can either be used in standalone approaches or in combinations (couplings) thereof (LC-MS and CE-MS).

Liquid chromatography: While reversed phase (RP), normal phase (NP), and high-performance anion-exchange chromatography (HPAEC) are nowadays the standard approaches for the separation and analysis of glycans, hydrophilic interaction chromatography (HILIC) with additional fluorescence detection (HILIC-FLR) is the most promising method for precision monitoring of glycans (Aich et al., 2016). For nearly all LC-based methods, the labeling of the sample with fluorescent dyes is required. Due to this preparation step, in-line or on-line PATs are not feasible, and automation of sampling, preparing, and chromatography after product formation is still challenging.

Mass spectrometry: Here matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is known to be the second most used standalone analytical method for glycan analysis. Although the quantitation of the product in the sample is limited, stability and automation possibilities make MALDI-MS a well-suited tool for precise analysis for glycan screening and product analysis of enzymatic synthesis (Harvey, 2021). Again, only at-line PATs seem feasible, making real-time monitoring of product formation impossible at this time.

Capillary electrophoresis: Although CE is well known for its separation power, and sensitivity, there are technical issues regarding reliability, stability, and reproducibility, thus up to now, CE is far less used than LC and MS-based methods. While these technical issues need to be resolved for common (usually single capillary) CE-instruments, combinations of CE with fluorescent measurements (CE-FLR) or laser-induced fluorescence (CE-LIF) are promising developments. In particular, multiplexed capillary gel electrophoresis with laser-induced fluorescent readout (xCGE-LIF), based on glyco-engineered DNA-sequencers, has been established and applied for HMOS (Kottler et al., 2013), and glycan analysis (Cajic et al., 2021; Hennig et al., 2016). The above-mentioned technical issues of CE are overcome and real high-throughput in high resolution and high sensitivity is now possible. As for LC, labeling glycan samples with fluorescent dyes is necessary. This again requires time-consuming sampling and preparation not suitable for real-time, but adaptable to at-line analysis of processes.

Emerging technologies: Since the enlisted methods all have their individual (dis-)advantages, it is of scientific effort to further develop a single method or combine the advantages of several methods into one. With that, new technologies for glycan analysis emerge as outlined by Rapp and colleagues (Pralow et al., 2021). At this point, porous graphitized carbon liquid chromatography coupled to MS (PGC-LC-MS) does not require glycan labeling and therefore allows faster analysis. Another promising method is ion-mobility mass spectrometry (IM-MS). Here, new technological developments allow further separation of glycans and thus more precise analysis. As it is obvious that nearly all of the enlisted methods require additional sample preparation, today's glycan analytics are not suitable for in-line or on-line PATs. Especially the establishment of an automated process for sampling and sample preparation is the most challenging aspect of AEGS (Fig. 10B). Importantly, analyses of products and intermediates were already included in some pioneering

work of automated glycan synthesis as reported in Chapter 4.

The automated dispenser apparatus, developed by the Boons group, contains a sample vial rack for analysis of intermediate products (Li et al., 2019a) (Fig. 9B). After each reaction cycle, a small sample is isolated from the reaction vessel for LC-MS analysis. The profiles of the products after each cycle give insights into glycan elongation towards the final product, for example, the elongation of poly-LacNAc after multiple cycles to obtain a decamer (**product 8 in** Fig. 4). However, the analysis of all reaction intermediates after each cycle is performed after the final step and completion of the target product. Real-time monitoring or intervention based on analysis data is therefore not possible. Nevertheless, the authors are aware of a system upgrade for the performance of follow-up steps directly depending on the analysis of intermediate products (Li et al., 2019a).

In the work of Gerardy-Schahn and colleagues meningococcal capsular oligosaccharides with an extensive degree of polymerization are produced (Fiebig et al., 2018) (Fig. 9D). Offline analysis of the products to define the length after different experimental parameters were conducted by Alcian blue/silver-stained PAGE and HPLC-anion-exchange chromatography (AEC). In addition to the analysis of the final product, the authors also included in-process monitoring to get information on the status of the reaction. Here, during the reaction process, samples were taken every 20 min and concentrations of UDP-GlcNAc and UMP were detected by HPLC analysis. From this data, the authors monitored the complete transfer of GlcNAc-1-phosphate to the nascent oligosaccharide, resulting in UMP as the only by-product in the reaction system (Fiebig et al., 2018). While the sole read-out of the by-product UMP is no replacement for a detailed investigation of the degree of polymerization of the final product, it can help to understand the process and allows a reasonable adjustment of reaction time.

Online monitoring in the immobilized microfluidic enzyme reactors (IMER) was achieved by connecting the reactor directly with an ESI-Q-ToF mass spectrometer (Heinzler et al., 2019) (Fig. 9C). Collection of the sample, preparation for the measurement, and transfer to the MS are automated processes and are conducted by a high-pressure multiport valve. Although not the whole process was monitored in real-time, samples were taken at important reaction time points, e.g. before starting a new synthesis cycle (Heinzler et al., 2019).

In conclusion, to take advantage of the sophisticated technologies in automated enzymatic glycan synthesis, the integration of precise analysis methods into the setup is essential. Monitoring is a critical benefit for process optimization in multistep/cycle glycan syntheses as well as for approval and commercialization of the products.

6. Outstanding questions

There remain still some outstanding questions, the list being not complete.

- Can synthetic strategies be combined for efficient synthesis and utilization and re-use of glycosyltransferases? For example, can the 'catch and release' strategy of products be combined with immobilized glycosyltransferases?
- What is the unifying strategy for the immobilization and reuse of glycosyltransferases?
- What are the catalytic efficiency and the optimized conditions of each immobilized glycosyltransferase to reach full substrate conversion in a short time?
- Is cascading of glycosyltransferases in a single reaction step possible for faster overall glycosylation?
- What is the best reaction performance - batch reaction versus continuous flow biocatalysis?
- How to monitor the glycan products in an automated mode with feedback control of the reaction?

7. Conclusions

In this review, we have highlighted strategies and concepts for automated enzymatic synthesis. An important prerequisite for the automation of enzymatic glycan synthesis is the immobilization of either the substrate or the enzymes. Different immobilization strategies have been established for enzymatic glycan synthesis. As seen, chemists prefer strategies focusing on product recovery - solid-phase synthesis with immobilized substrates or 'catch and release' of products, whereas researchers with a biotechnological background prefer synthetic strategies using immobilized enzymes and re-use of the valuable biocatalysts. A versatile library of different enzymes for glycan synthesis has been established by the scientific community, offering specific enzyme sets for each target structure.

However, the choice of one strategy is not easy to take when it comes to the construction of an apparatus for consumers being not experts in the field of synthetic glycobiology and enzyme technology. Less complex glycans have already been synthesized in an automated approach with enzymatic bioreactors. Here, different strategies based on glycan- or enzyme immobilization, compartmentation to different reaction compartments, or filter- and retention mechanisms have been established. Close monitoring of the synthesis for optimal regulation of reaction parameters is essential to fully take control of the process. Now it is the time to transfer and combine this knowledge towards a ubiquitous platform for automated enzymatic synthesis of a wide variety of glycans. Reaching this goal is an interdisciplinary endeavor, complementing expertise in carbohydrate chemistry, enzyme technology, reaction engineering, analytical chemistry, and data analysis.

Author contributions

L.E., M.F., A.P., and E.R. conceived the project scope. L.E., K.P.H., and P.P. performed the literature search. K.P.H. and P.P. designed the figs. K.P.H., P.P., and L.E. drafted the manuscript, all authors contributed to the discussion, editing, and completion of the manuscript and all authors approved the final submitted version.

Credit author statement

Kai Philip Hussnaetter and Philip Palm contributed equally.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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