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REVIEW

Small tools for sweet challenges: advances in microfluidic technologies for glycan synthesis

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Abstract

Glycans, including oligosaccharides and glycoconjugates, play an integral role in modulating the biological functions of macromolecules. Many physiological and pathological processes are mediated by interactions between glycans, which has led to the use of glycans as biosensors for pathogen and biomarker detection. Elucidating the relationship between glycan structure and biological function is critical for advancing our understanding of the impact glycans have on human health and disease and for expanding the repertoire of glycans available for bioanalysis, especially for diagnostics. Such efforts have been limited by the difficulty in obtaining sufficient quantities of homogenous glycan samples needed to resolve the exact relationships between glycan structure and their structural or modulatory functions on a given glycoconjugate. Synthetic strategies offer a viable route for overcoming these technical hurdles. In recent years, microfluidics have emerged as powerful tools for realizing high-throughput and reproducible syntheses of homogenous glycans for the potential use in functional studies. This critical review provides readers with an overview of the microfluidic technologies that have been developed for chemical and enzymatic glycan synthesis. The advantages and limitations associated with using microreactor platforms to improve the scalability, productivity, and selectivity of glycosylation reactions will be discussed, as well as suggested future work that can address certain pitfalls.

Keywords Bioanalytical methods · Biochips/high-throughput screening · Biopolymers/lipids · Microfluidics/microfabrication · Biotechnological products · Enzymes

Abbreviations

PTMs	Post-translational modifications
ER	Endoplasmic reticulum
GA	Golgi apparatus
glycoenzymes	Glycan active enzymes
AGA	Automated glycan assembly
CAZy database	Carbohydrate-Active Enzyme database
GTase	Glycosyltransferase
GH	Glycosidase/glycosyl hydrolase
GS	Glycosynthase
OPME	One-pot multi-enzyme
FLPE	Fluorous liquid phase extraction
$TMSB(C_6F_5)_4$	Tetrakis(pentafluorophenylborate)
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
MUC1	Mucin-1
IEMR	Immobilized enzyme microreactor
SPase	Sucrose phosphorylase
CGTases	Cyclodextrin glycosyltransferases
APTES	3-Aminopropyl triethoxysilane
CSS	CMP-sialic acid synthetase
ISMR	Immobilized substrate microreactor
SLB	Supported lipid bilayer
GFP	Green fluorescent protein

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Introduction

Post-translational modifications (PTMs) play an integral role in modulating the biological functions of macromolecules. Glycosylation, wherein carbohydrates are covalently linked to lipids or proteins, is one of the most ubiquitous PTMs [1–6]. For individual molecules, changes in their glycosylation status can result in altered biological function [7, 8].

Given glycosylation's integral role in various biological processes, it is not surprising that several disease pathologies correlate with variations in glycosylation patterns [1–6]. The importance of glycans in human health is underscored by the increasing prevalence of glycoconjugate biotherapeutics, particularly recombinant monoclonal antibodies. Between 2015 and 2018, glycosylated monoclonal antibodies made up 57% of FDA-approved biotherapeutics [9]. Thus, understanding the relationship between glycan structure and function is critical to identifying and engineering better glycan-modified therapeutics; however, these efforts are stymied by the difficulty in synthesizing complex glycans structures.

Early efforts in glycobiology relied on isolating natural glycans primarily from animal tissue. A persistent bottleneck in natural glycan isolation arises from glycosylation being a non-templated process that occurs in the endoplasmic reticulum (ER) and Golgi apparatus (GA). These organelles house the resident glycan active enzymes (glycoenzymes), which act in cohort to assemble oligosaccharide chains onto various biomolecules. Natural variability in the expression of glycoenzymes, substrate concentration, and co-factor availability give rise to heterogeneity in natural glycans. A single glycoprotein can exhibit multiple glycoforms, with variation in the site(s) and extent of glycosylation, as well as the composition of the distal glycan moiety. This heterogeneity in glycan structure and linkage makes routine isolation of homogenous glycoforms in sufficient quantities nearly impossible. Consequently, research in glycoscience within recent years has largely focused on developing scalable methods to access and prepare structurally defined glycans on-demand.

Microfluidic technology has emerged as a promising tool for realizing high-throughput preparations of homogenous glycans. Microfluidic devices feature micron-sized dimensions and operate under continuous fluid flow. These features make microfluidics appealing for several reasons. The small dimensions and flow conditions can enhance mass transport and, by extension, reaction kinetics. The small volumes can reduce the time and quantity of reagents required for process optimization, minimizing costs and, in some cases, safety hazards. Micro-structured platforms are highly modular, allowing easy integration of multiple processing units in series and in parallel. This last feature makes microfluidics particularly well-suited for process automation and scale-up. Over the past two decades, chemists have leveraged microfluidics to develop more efficient methods for the preparation of diverse glycan standards.

This review explores emerging trends in the development of microfluidics for the synthesis of chemically defined oligosaccharides and biological glycoconjugates, including glycolipids and glycoproteins. The "State-of-the-art methods for glycan synthesis" section provides an overview of current state-of-the-art techniques for glycan synthesis, including chemical and enzymatic methods. The "Overview of microfluidics" section discusses the utility of microfluidics for addressing major challenges undermining conventional techniques. The "Microfluidic technologies for chemical glycan synthesis" section delves into recent applications of microfluidics in chemical glycan synthesis, and the "Microfluidic technologies for enzymatic glycan synthesis" section explores the parallel pursuit of microfluidic platforms for enzymatic glycan production. Special attention will be given to the advantages that micro-structured devices offer in streamlining the synthesis of glycosylated molecules. The limitations of existing platforms will also be presented with illustrative examples of the challenges still faced in the field of glycan synthesis. A summary of the major technological developments mentioned in each section is provided in Fig. 1.

State-of-the-art methods for glycan synthesis

Overview

As more is learned about the roles glycans play in biology, so grows the demand for ready access to chemically defined glycans. However, synthesizing homogenous samples of structurally defined glycans remains a formidable task in carbohydrate chemistry. Early work in this area focused on developing chemical tools to control regio- and stereochemistry in glycosylation reactions. The interdisciplinary nature of glycobiology later led to the creation of more tractable techniques, such as enzymatic strategies and hybrid chemoenzymatic methods. Within the past decade, advances in automated chemical and biocatalyst-based approaches have made glycan synthesis more accessible to non-specialists. The following sections highlight key synthetic methods that have contributed to the field of glycomics, as well as the persistent challenges faced with these approaches.

Chemical strategies

Chemical glycosylation has emerged as a primary strategy for preparing homogenous glycan samples.

During chemical glycosylation, a glycosyl donor transfers a glycan, which acts as an electrophile, to a glycosyl acceptor, which acts as a nucleophile, in the presence of a promoter (i.e., a catalyst). Glycans are composed of monosaccharides interconnected by glycosidic bonds, wherein the anomeric carbon of a sugar monomer is linked to a hydroxyl group on another compound, such as a sugar, amino acid residue, or lipid. When one or more glycosyl moieties are linked to a non-carbohydrate (aglycone), the glycan is referred to as a glycoconjugate. This review will focus on

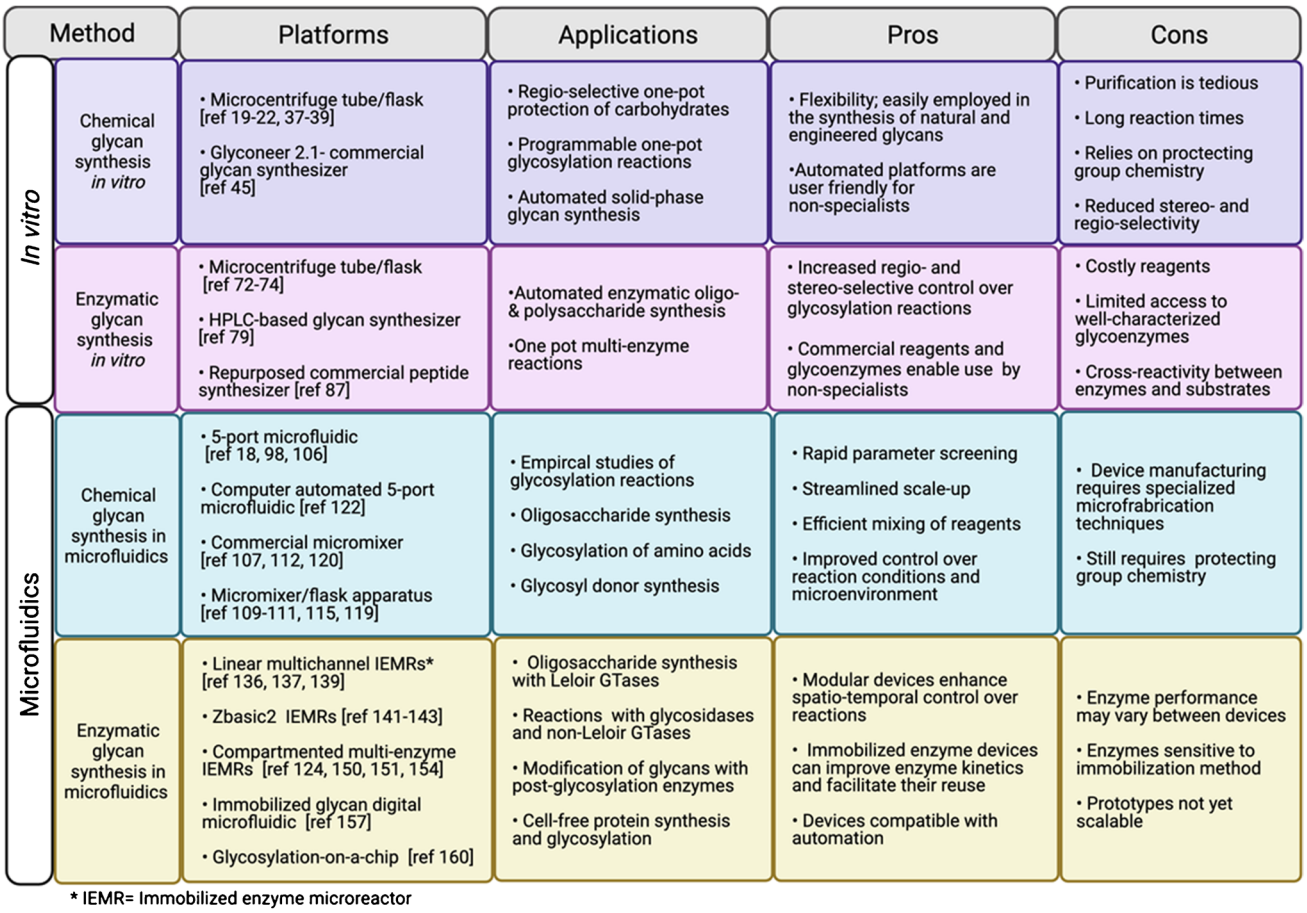


Fig. 1 Summary of major technological developments for glycan synthesis. Created with BioRender.com

select glycoconjugate types, including glycoproteins, glycolipids, and proteoglycans such as glycosaminoglycans (Fig. 2a). Glycosidic bonds can form as α- or β- linkages depending on the reaction mechanism (Fig. 2b). Additionally, as polyhydroxyl compounds, each monosaccharide contains multiple sites for glycan attachment. Thus, synthesizing specific carbohydrate structures requires regio- and stereoselective control over glycosidic bond formation.

Considering these requirements, the goal of many chemical methods is to design glycosyl donors, glycosyl acceptors, and promoter systems that optimize the regio- and stereoselective yields of glycosylation reactions. Selection of the leaving group on the glycosyl donor is a key strategy by which chemists modulate regio- and stereochemical outcomes. Popular leaving groups include O-glycosyl imidates, especially tricholoracetamidates and trifluoroacetamidates [10, 11], and a plethora of alternate strategies have been developed over the years, including O-glycosyl halides, thioglycosides, and O-glycosyl phosphates [12–18]. The addition and manipulation of protecting groups on acceptors and donors offers another means by which chemist achieve regio- and stereochemical control in glycosylation reactions. Protecting groups are chemical moieties that shield hydroxyl groups and prevent their reaction with other reagents. Glycosidic bonds can thus be formed in a site-specific manner at unprotected hydroxyls. In serial reactions, protecting groups may be added as persistent moieties that remain intact throughout the synthesis or as temporary groups that are strategically removed at intermediate steps. However, protecting group strategies are time-consuming endeavors that typically require isolation and purification of substrates after each modification. Hung and coworkers expedited carbohydrate protection by developing a regio-selective one-pot protection procedure wherein glycosyl donors are equipped with orthogonal protection groups of varying reactivity [19, 20]. This method enables multiple protection/deprotection steps to proceed in a single volume without the need to isolate intermediate species. Early protocols required per-*O*-silylation of sugars prior to one-pot protection, but recent advances now allow the use of unprotected sugars as starting materials [21, 22].

Stereodirecting of glycosylation reactions is often achieved through the placement of participating protecting groups at the neighboring C-2 position or at remote

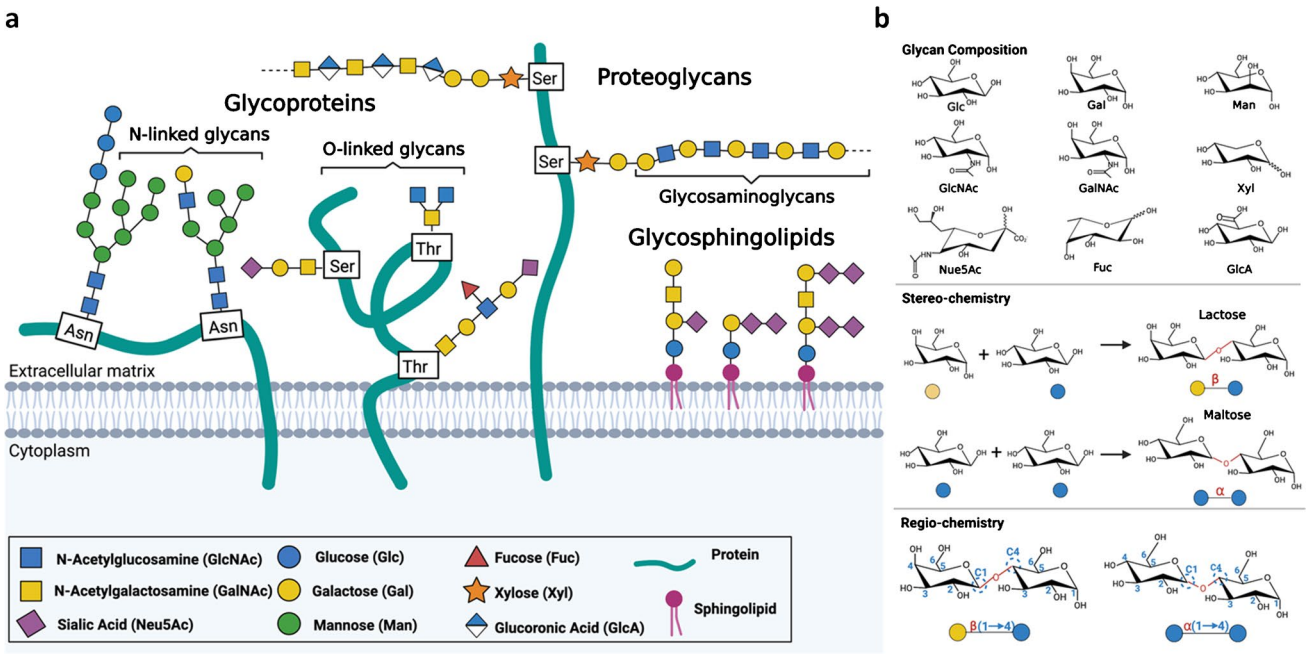


Fig. 2 (**a**) Major glycan and glycoconjugate classes found in humans. The glycoconjugate classes shown here include N- and O-linked glycoproteins. N-linked glycoproteins contain glycan chains (N-glycans) that are covalently linked to asparagine (Asn) residues in the protein. In O-linked glycoproteins, glycan chains (O-glycans) are covalently linked to serine (Ser) or threonine (Thr) residues. Proteoglycans consist of a core protein linked to one or more polysaccharide chains known as glycosaminoglycans. Glycosphingolipids are comprised of a membrane-bound sphingolipid tail and a sialylated glycan head group. (**b**) Multiple factors contribute to glycan heterogeneity. Human glycans have diverse glycan compositions which arise from the nine monosaccharide building blocks used in their assembly. The configuration (stereochemistry) and location (regiochemistry) of glycosidic bonds also vary. Glycosidic bonds formed between two sugar monomers can adopt a β or α configuration such as that shown for lactose and maltose, respectively. Linkage formation occurs at the anomeric carbon (C1) of the donor monosaccharide and any free hydroxyl group on the acceptor monosaccharide, amino acid residue (not shown), or lipid (not shown). For both lactose and maltose, the glycosidic bond links the C1 carbon of the donor to the C4 carbon of the acceptor. Created with BioRender.com

carbons of the glycosyl donor [23, 24]. These protecting groups interact with the oxocarbenium cation intermediate generated during the glycosylation reaction. This interaction causes the formation of a more stable dioxolenium ion, which is shielded by the participating group on one face from nucleophilic attack. Thus, glycosidic bonds selectively form on the face opposite of the participating moiety. Neighboring group participation favors the formation of 1,2-trans glycosidic linkages while remote group participation renders 1,2-cis-glycosidic linkages [25–29]. Stereo-control is also achieved by regulating donor reactivity through the armed/disarmed principle, which stems from observations that certain protecting groups reduce donor reactivity ("disarming") relative to other protecting groups ("arming") [30]. "Arming" and "disarming" groups are widely used to control glycosylation through selective activation of glycosyl donors. In particular, the armed/disarmed concept has enabled chemists to perform serial glycosylation reactions without needing to isolate intermediates as discussed below. We encourage readers to consult recent reviews for detailed discussions on the application of participating groups in stereoselective carbohydrate synthesis [23, 24].

Several solution-phase and solid-phase synthesis methods have made the chemical preparation of glycosides more practical. A key technical development in solution-phase chemistry was the creation of one-pot multistep glycosylation, wherein multiple reactions are carried out sequentially in a single reaction vessel [31]. In these schemes, glycosyl donors are designed to exhibit varying reactivities to control the order of their participation in oligosaccharide assembly. One approach to one-pot glycosylation uses the armed/disarmed principle to tune donor reactivities via their protecting group substituents. Alternative approaches exist which bypass protecting group manipulation, including orthogonal one-pot systems wherein donor participation is regulated by varying the leaving group [32]. Additionally, a pre-activation method has also been developed, wherein donors are selectively activated through exposure to a stoichiometric amount of a suitable promoter [33]. Researchers have leveraged one-pot multistep procedures to assemble a broad range of complex human, pathogenic, and therapeutic glycans [34, 35]. Indeed, the largest oligosaccharide assembled with one-pot

methods was a 92-mer arabinogalactan [36]. However, purification demands for one-pot syntheses scale with glycan size, making routine synthesis of complex glycan structures a tedious task with solution-phase approaches.

Computer-aided approaches known as "programmable one-pot" systems have helped automate the design of one-pot protocols, which rely on careful selection of monosaccharide building blocks and their leaving and protecting groups [37, 38]. These methods typically make use of software programs that catalogue and compare reactivity data collected for diverse glycosyl donors and acceptors. Wong and coworkers were the first to demonstrate this concept through the creation of the Optimer program, a searchable database of 50 thioglycoside glycosyl donors and their empirically determined reactivity values [37]. Users of Optimer can input a desired glycan structure and receive a recommendation for combinations of donors needed to synthesize the target glycan. To facilitate the synthesis of more complex and diverse oligosaccharides, Wong et al. created an updated program, Auto-CHO, which contains an expanded library of experimentally validated thioglycoside donors and incorporates a machine learning algorithm to predict reactivity data for thousands of other donors [38]. Using Auto-CHO, the authors were able to synthesize several oligosaccharides, including Globo H and heparin pentasaccharide, with higher yields than previously reported. More recently, Wong and Wang et al. developed the GlycoComputer program, which predicts the yield and stereoselectivity of a glycosylation reaction using reactivity data from donors and acceptors [39]. In general, computer-assisted methods have begun to reduce the amount of trial and error required to develop one-pot protocols.

Advances in solid-phase techniques have helped chemists circumvent purification demands characteristic of solution-phase synthesis. In solid-phase reactions, the glycosyl donor or acceptor is tethered to a solid support, allowing the isolation of products from reagents or undesired by-products to be streamlined through simple wash steps. Popular techniques include polymer-supported synthesis and automated glycan assembly (AGA). In polymer-supported syntheses, glycosyl acceptor or donor substrates are immobilized onto a soluble or insoluble polymer support [40, 41]. Using carefully designed monosaccharide building blocks, oligosaccharide assembly can proceed from the nonreducing end to the reducing end of the glycan chain (immobilized donor), the reverse direction (immobilized acceptor), or in a bidirectional fashion [42, 43]. The simplicity of solid-phase reactions makes them highly compatible with automated workflows. Seeberger and colleagues were the first to demonstrate AGA by developing an automated solid-phase glycan synthesizer [44]. This was followed by the creation of the commercialized Glyconeer system, which incorporates resin bound oligosaccharide acceptors [45]. Other automated synthesizers have since been fabricated including an HPLC-assisted platform, fluorous-tag-assisted methods, and an electrochemical-based platform [46–48]. These technologies have facilitated the synthesis of a broad range of glycans with varying degrees of complexity, including glycosaminoglycans, an over 100-mer polysaccharide, and glycopeptides [49–51]. Even still, broader use of AGA platforms has been limited by the lack of a universal procedure that can be readily applied to any glycan target. AGA systems still rely on protecting group chemistry, thus the design of the glycosyl donors and acceptors must be tailored for each glycan target. Stereoselective formation of challenging linkages, such as 1,2-*cis* linkages and β-mannosides, remains elusive with AGA, although advances in protecting group characterization and design are beginning to change this [28, 29, 52, 53].

Chemical glycosylation has proven to be a crucial tool for generating chemically defined glycans in lab settings. Chemical methods afford researchers excellent flexibility as they allow for the creation of virtually any glycosidic linkage. Through careful schematic design, researchers have reproduced countless linkages and oligosaccharide chains found in nature and many more glycans containing non-natural linkages and compositions. Moreover, analogs of biological saccharides and glycoconjugates can be readily engineered with chemical strategies. This is apparent in the growing use of the chemically prepared glyco-therapeutics, such as carbohydrate antibiotics, vaccines, and therapeutic glycoproteins [54]. Nevertheless, many chemical techniques remain intractable to non-specialists. Conventional techniques still draw upon advanced protection and de-protection techniques to achieve stereochemical and regiochemical control of reactions. Consequently, many reaction schemes require tedious purification procedures, limiting the size of molecules achievable in vitro. While solid-phase reaction platforms have addressed some of these issues, some glycan modifications remain out of reach without using alternative synthetic approaches.

Enzymatic and chemo-enzymatic strategies

Chemists and biologists have managed to overcome many of the challenges presented by chemical preparation of glycans by leveraging the biological machinery that cells use to produce these complex molecules. Recent advances in recombinant protein production have led to the growing use of glycan-modifying enzymes in carbohydrate synthesis. Genes encoding glycan-active enzymes are being identified at an exponential pace and catalogued in open-source databases, such as the Carbohydrate-Active Enzyme (CAZy) database, to facilitate knowledge transfer across labs and fields [55]. Current entries indicate that the human genome encodes approximately 300 enzymes that catalyze the extension, cleavage, or modification of glycans, and thus provide a rich

toolbox for the selective, biocatalytic synthesis of glycosides [56]. Improvements in heterologous and cell-free expression systems have expanded the number of such enzymes that can be recombinantly produced for in vitro applications [57]. Glycoenzymes offer an attractive alternative to chemical methods, as biocatalysis proceeds under physiological conditions in a stereo- and regioselective manner, eliminating the need for tedious protection and deprotection steps as well as harsh reaction conditions.

Three types of glycoenzymes are commonly employed in glycoside synthesis: glycosyltransferases (GTases); glycosidases, also known as glycosyl hydrolases (GHs); and enzymes that catalyze post-glycosylation modifications. GTases catalyze glycan extension by transferring monosaccharides from sugar donors to acceptor molecules. Two subtypes of these enzymes exist in nature: non-Leloir and Leloir GTases. Non-Leloir GTases, also known as phosphorylases, utilize disaccharides (e.g., sucrose), glycopolymers (e.g., starch), and phosphorylated sugars as glycosylation donors. Phosphorylases are not as well-studied as other glycoenzyme classes due to their poor synthetic potential. Competition between the phosphorolytic and synthetic activities of these enzymes limits the yield of synthetic product unless reaction conditions are precisely biased in one direction [58, 59]. However, efforts to investigate and engineer phosphorylases for industrial-scale synthesis have grown in recent years owing to the low cost and wide availability of their donor substrates, as well as their inherent tolerance of diverse acceptor molecules [59]. Leloir GTases utilize nucleotide-activated monosaccharides as donor substrates. Sugars activated by uridine diphosphate, such as UDP-galactose (UDP-Gal), and cytidine monophosphate, such as CMP-sialic acid (CMP-Sia), occur in mammalian systems. Due to the high costs of nucleotide sugar substrates, Leloir enzymes have mostly been applied to the production of high-value glycosides, particularly pharmaceuticals, where high and selective product yields are essential. A more extensive description of the functionalities and applications of non-Leloir and Leloir GTases can be found in several reviews [59–62].

GHs catalyze the hydrolysis of glycosidic linkages, as well as the reverse condensation reaction, to synthesize glycosidic bonds. Monosaccharides, oligosaccharides, and non-activated sugars can serve as the glycosyl donors. GHs cleave at terminal glycosidic bonds (exo-glycosidases) to release single monosaccharides or at internal glycosidic bonds (*endo*-glycosidases) to release di- or oligosaccharide moieties. These enzymes offer appealing synthetic tools because of their wide commercial availability, inexpensive donor substrates, and compatibility with organic solvents [63]. However, GH-catalyzed reactions are prone to low yields caused by product hydrolysis. Researchers have circumvented this issue by mutating GHs to favor synthetic activity. These glycosynthases (GSs) were first generated by Withers and coworkers in 1998 through mutagenesis of a *Agrobacterium* sp. β-glucosidase and have since been derived from various endo- and exo-glycosidases [64–66]. Several groups have published extensive reviews on the engineering and applications of GH and GS enzymes for glycoside synthesis [66–69].

Enzymes that modify sugar residues within carbohydrate chains during or after glycosylation comprise a third major class of biocatalysts employed in glycan synthesis [70]. Chemical modifications such as sulfation, o-acetylation, and phosphorylation often occur at carbohydrate hydroxyl groups and are known to modulate glycan function. As a result, there is a growing desire to synthesize these substructures and link them to biological functions. Most attempts to reproduce these modifications have relied on enzymatic and chemo-enzymatic methods and have been limited to modifications achievable using widely available enzymes. As a result, most work has focused on sulfated glycans, especially GAGs, owing to the popularity of sulfotransferases and sulfatases [71]. Advances in the identification and recombinant expression of glycan-modifying enzymes are needed to better understand the biological significance of glycan modifications and to realize their potential as synthetic tools.

Of the three types of glycoenzymes mentioned, GTases have become the enzyme of choice for glycan synthesis. Eukaryotic GTases demonstrate high specificity for their cognate donor and acceptor substrates, making them well-suited for one-pot methods. Many of the sugar nucleotides found in humans are now commercially available, making such reactions feasible to perform by non-specialists. However, the high cost of sugar nucleotides limits the scale of enzymatic one-pot synthesis reactions. The recent development of sugar-nucleotide generation systems, wherein donor substrates are enzymatically generated in situ from simple monosaccharide and nucleotide starting material, has minimized the costs associated with GTase-driven synthesis. Accordingly, one-pot multi-enzyme (OPME) reaction systems, which combine GTases with sugar nucleotide generation systems, have become a popular strategy for enzymatic glycan production [72–75]. Solid-phase methods that employ glycoenzymes are particularly useful for high-throughput preparations of glycan libraries, such as those used in widely applied carbohydrate microarrays [76]. Both immobilized substrate and immobilized enzyme platforms have been developed for this purpose. Anchoring substrates is often preferred since products are easily isolated with simple wash steps, making the procedure itself amenable to automation [77, 78]. Substrate immobilization can occur before or after glycan synthesis using a wide variety of support materials such as a resin, polymer, or solid surface [79–83]. On the other hand, immobilized enzyme platforms have been implemented sparingly to carry out enzymatic

glycoside syntheses [84, 85]. While enzyme immobilization can facilitate the recovery and reuse of high-value biocatalysts such as GTases, the inability to capture target products from reaction mixtures makes the automation of these platforms less straightforward.

The automation of enzymatic glycan synthesis is still in its infancy; however, several labs have shown progress in realizing automated platforms. The earliest demonstration was reported by Nishimura and coworkers in 2010 [79]. Their platform consisted of an HPLC-based glycan synthesizer and utilized polymer-supported glycosyl acceptors alongside recombinant GTases to generate a sialyl Lewis oligosaccharide. The process took 4 days and provided a 16% product yield. Seeberger and colleagues subsequently combined AGA methods with enzymatic sialylation to semi-automate the production of sialyloligosaccharides, which are notoriously difficult to render by chemical means alone [86]. The Wang lab recently realized the first fully machine-driven system for the automated enzymatic synthesis of oligo- and polysaccharides [87]. The platform was constructed by repurposing a commercial peptide synthesizer and employed polymer-supported precursors and a recombinant bacterial GTase to generate a panel of glycan structures, including GM1 pentasaccharide, blood group antigens, and poly-N-acetylactosamine.

Several challenges remain in leveraging enzymatic and chemo-enzymatic approaches in glycobiology. For one, access to glycan active enzymes remains limited. Only a small fraction of the glycan active enzymes reported in the CAZy database have been functionally characterized, and even fewer have undergone structural characterization [55]. Consequently, many glycosidic linkages cannot yet be recapitulated in vitro with enzymatic methods. Low orthogonality between activated sugar donors and acceptors has also limited the number of glycosylation reactions that can be performed in one-pot systems. For GTase-catalyzed reactions in particular, feedback inhibition continues to be an issue. While nucleotide sugar generation systems have been used to mitigate this effect, many of these systems are also enzymatically driven and limited by low enzyme activity and small substrate concentrations [88]. Automated enzymatic methods could help overcome these drawbacks, but successful attempts have been low-yielding and rely upon specialized equipment, large reaction volumes, and long reaction times. Simple and efficient systems for large-scale enzymatic glycoside synthesis remain a work in progress.

Overview of microfluidics

Microfluidics are micron-sized platforms wherein fluid flow is precisely controlled. These devices are often constructed as narrow channels supported by or etched into solid substrates with dimensions that range between 100 nm to 100 µm [89]. The design of microflow systems encompasses a broad spectrum of materials, architectures, and flow patterns. The most common materials used in the fabrication of microfluidics include silicon, glass, stainless steel, and polymers, especially polydimethylsiloxane (PDMS) [90]. Three types of architectures have proven particularly popular across fields: open-tubular devices, packed bed systems, and monolith-containing platforms. Unique device classes have also arisen in the past decade that feature distinct flow modalities. Among them are droplet-based devices, digital microfluidics, and inertial microfluidics, which have all been recently reviewed [91–93]. Additionally, many of these platforms incorporate active components such as valves, mixers, and pumps to enable precise regulation of the flow rates and paths [94–96].

The versatility of microfluidics is reflected in their ever-growing use by laboratories spanning a broad range of scientific disciplines. Applications of microfluidics have predominantly aimed to improve the performance of macro-scale analytical procedures. Process miniaturization offers several advantages in this regard (Fig. 3). The small dimensions of microfluidics greatly reduce reagent and sample consumption, while also affording users precise control over the analytical microenvironment. The small length scales also promote efficient mixing of fluids, rapid heating and cooling, as well as enhanced mass transfer, which often translates into more efficient and sensitive processes. Accordingly, microfluidics have garnered widespread use in high-throughput screening of conditions for biological assays and chemical transformations [97, 98]. Another notable quality of microfluidics is their ability to integrate multiple processing units in sequence or in parallel [99]. Microfluidic designs that afford inline separation and detection of analytes have been developed, such as the miniaturization of electrophoresis and its coupling to mass spectrometry [100]. This compatibility with multiplexing also makes microfluidics well-suited for automation. Because the small length scales mirror the dimensions of living systems, microfluidics have become popular tools for cell-culture and examining single cells and individual molecules [101–103].

Though slower to adopt microfluidic systems, laboratories that specialize in chemical synthesis have demonstrated the utility of microreactors in enhancing synthetic protocols. In addition to the advantages listed above, miniaturizing reactions can improve the overall safety of chemical processes by reducing the volume of harsh reagents as well as the risk of thermal runaway in highly exothermic reactions. The benefits of microfluidics also extend to the scale-up of reactions, as production volume can be readily increased by running devices for longer periods of time (i.e., "scale out" principle) or by increasing the number of reactors run in parallel (i.e., "numbering up") (Fig. 3b). Consequently, the

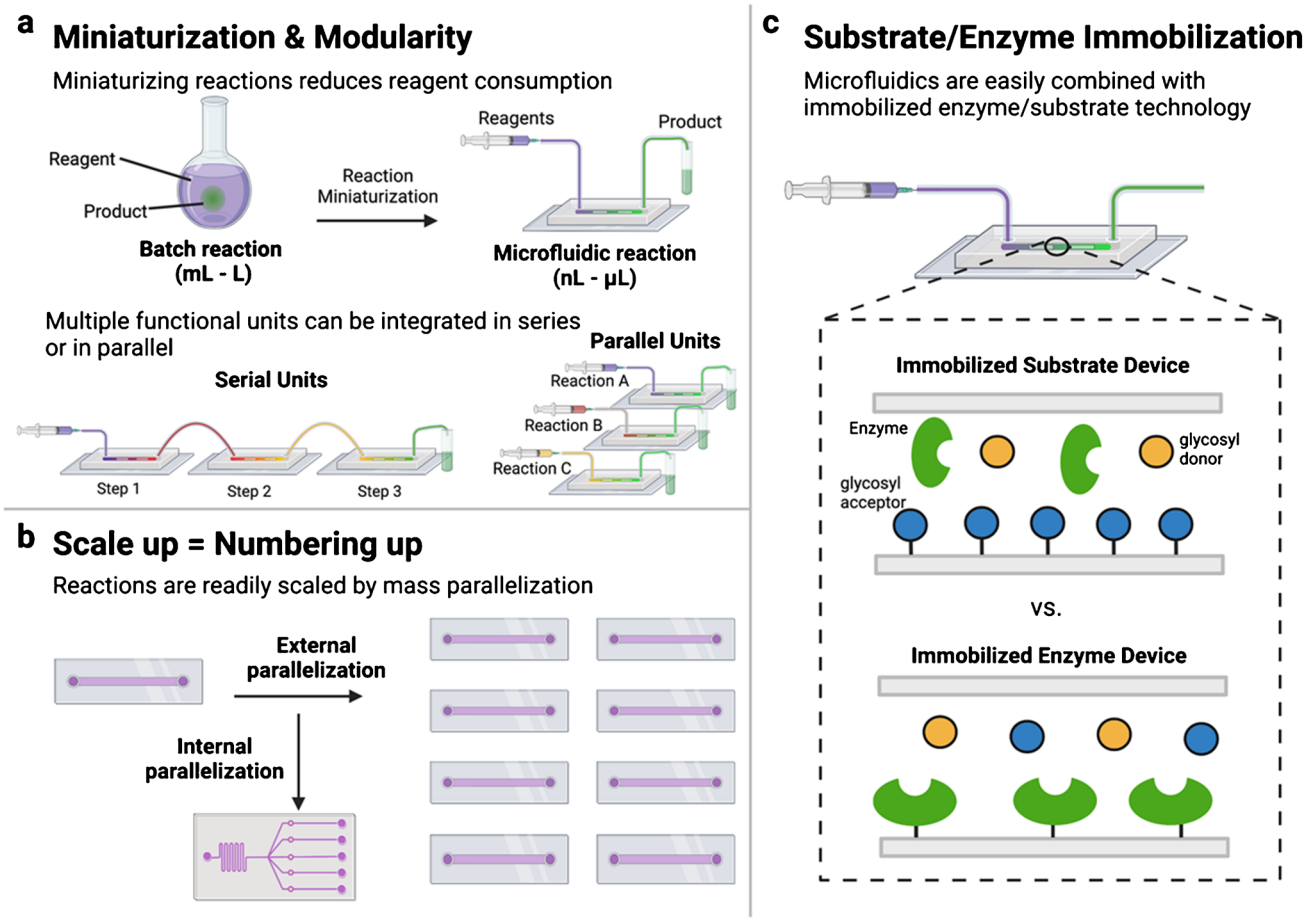


Fig. 3 An illustration of major advantageous features of microreactor systems. (**a**) Microfluidics significantly reduce the reaction volumes from the milliliter/liter scale down to nanoliter/microliter volumes. Microfluidics are modular platforms that allow for multiple processing units to be integrated in series or in parallel, allowing for increased throughput capacity. (**b**) Microfluidic reactions are easily scaled using the "numbering up" principle where microchannels are parallelized in a single device (internal parallelization) or across multiple devices (external parallelization). (**c**) Microflow systems are often designed as immobilized substrate platforms to streamline purification of intermediates and products or as immobilized enzyme platforms to facilitate the recycling of enzymes. Created with BioRender.com

conditions for analytical and preparative scale reactions are easily kept identical and the time and resources ordinarily required to optimize reactor designs in conventional scale-ups are reduced if not eliminated [104].

As tried-and-true solution-phase and solid-phase methods continue to evolve, carbohydrate chemists have simultaneously borrowed from flow chemistry to expand their synthetic toolbox. Table 1 highlights signature features of microflow systems which can directly address key challenges in the field of glycobiology that have been elusive with traditional solution-phase and solid-phase chemistry. Foremost is the need for more efficient routes to prepare diverse catalogs of structurally defined glycans. Solution-phase reactions performed in flasks are simple to implement and have served as convenient tools to devise novel reaction schemes, while solid-phase methods have been instrumental in realizing large-scale automated syntheses. However, both approaches currently require characteristically long processing times. Furthermore, the yields and selectivity of chemical transformations are still tightly linked to the careful design of saccharide building blocks and fine-tuning of reaction conditions, which until recently were largely developed by trial and error. Consequently, an overreliance on batch methods has often hindered the optimization and scale-up of glycosylation reactions. The use of microflow systems and their combination with batch methods could help chemists overcome these bottlenecks in glycan production for glycomics research.

Because synthetic glycans offer the most viable path towards decoding the human glycome, technologies that enhance the speed and reproducibility of glycan synthesis stand to substantially advance our understanding of the link between glycan structure and biological function. The following sections highlight the ways in which microfluidic

Table 1 A comparison of the three major synthetic approaches to glycan synthesis

Synthetic method	Features	Advantages	Disadvantages
Solution-phase synthesis	- Widely used in chemical and enzymatic glycosylation reactions - Acceptor(s), donor(s), (bio)catalyst(s), and other additives are combined in a single reaction vessel for single-step or multi-step reactions (e.g., one-pot synthesis)	- Simple to implement - Convenient for developing new transformations - Computer-assisted methods (e.g., Optimer and Auto-CHO) facilitate rational design of one-pot protocols	- Time-consuming to optimize - Relies on protecting group chemistry for chemical transformations - Slower mixing leads to local variation in concentration and less predictable outcomes - More variability observed between reactions - Often requires tedious multi-step purifications to remove reagents and side products - Syntheses can take hours to days to complete
Solid-phase synthesis	- Predominantly used in chemical glycosylation reactions - Acceptor or donor linked to solid support - Requires suitable, orthogonal protecting groups and linker system for chemical transformations	- Amenable to automation and commercial systems available (e.g., Glyconeer 2.1) - Allows rapid generation of small molecule libraries - Facilitates synthesis of longer polymers (e.g., 50-mer oligosaccharides) - Contaminants removed by simple wash steps, minimizing purification demands	- Relies on protecting group chemistry for chemical transformations - Challenging to install α-glycosides - Purification of deprotected final products can be difficult - Syntheses still take hours to days to complete
Flow microreactors	- Prototypes for chemical and enzymatic glycosylation reactions have been reported - Tube or channel-like devices with micron-sized dimensions - Modular platforms - Reagents combined by pumping fluids into device via a reagent delivery system	- Convenient for scale-up or screening reaction conditions - Efficient heat and mass transfer facilitates fast mixing - Precise control over reaction time and temperature - Highly amenable to automation - Reduces reagent consumption and thus process costs - Compatible with inline/online analytics	- Microfabrication require access to specialized equipment and expertise - Process development can be time-consuming

systems have helped scientists' rapidly access, optimize, and scale diverse glycosylation reactions using chemical, enzymatic, and chemo-enzymatic methods. Special attention is given to the role these platforms have played in improving the homogeneity and yield of target glycan products as well as the efficiency of the glycosylation reactions relative to conventional in vitro approaches.

Microfluidic technologies for chemical glycan synthesis

Single-step synthesis of di- and oligosaccharides

Optimizing chemical reactions is one of the most formidable steps in chemical glycosylation. Once optimal conditions are identified at bench-scale, process scale-up presents additional challenges as reaction parameters must often be re-adjusted for larger volumes. Continuous flow microreactors have emerged as advantageous tools for circumventing these issues during process development. The application of microfluidics to chemical glycosylation methods has been pioneered largely by the labs of Prof. Peter H. Seeberger and Prof. Koichi Fukase. Over the past two decades, both of their groups have leveraged microfluidics to optimize various carbohydrate syntheses, the first demonstration of which was reported by Seeberger and colleagues in 2005 [98]. In this seminal work, the group used microfluidics to carry out α-mannosylation reactions, one of the most challenging chemical transformations in oligosaccharide synthesis [105]. The team fabricated a silicon-based device consisting of three inlet ports that converged into a serpentine microchannel that was partitioned into a short mixing zone and a longer reaction zone terminated by an outlet stream (Fig. 4). A fifth port was integrated towards the end of the reaction zone to deliver quenching agents. The reduced processing times and volumes required for each experiment enabled the authors to screen 44 reaction conditions, at varying temperatures and times, with a single preparation of reagents. Under the optimized microreactor conditions, stereoselective production of a mannosylated disaccharide proceeded within minutes, a significant improvement over the hours-long procedure reported for analogous batch reactions.

Since this first demonstration, microfluidics have mostly been leveraged for high-throughput screening of reaction parameters. The earliest applications focused on single-step transformations involving glucosylation and more challenging glycosidic transformations, particularly α-sialylation and β-mannosylation [106, 108–111]. Fukase and colleagues were instrumental in devising strategies to improve the yields and stereoselectivity of α-sialylation through microfluidic approaches. Natural sialic acid linkages participate in a range of biological interactions due to their prevalence

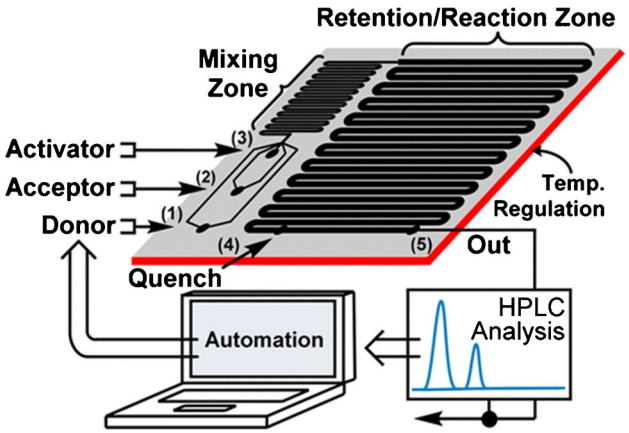


Fig. 4 Recent examples of microfluidic systems developed for chemical glycosylation reactions. (**a**) A schematic of the automated glycosylation instrument designed by Seeberger and coworkers. The system consists of a five-port silicon-based microfluidic, which was developed and used in previous work by the group [18, 98, 106]. The device is coupled to HPLC for in-line analysis and automated by computer software [107]. Reproduced from reference 107 with permission from the American Chemical Society

as terminal moieties on biological glycoconjugates. Consequently, the reliable and practical synthesis of natural sialic acid linkages, particularly the α(2,3)-linked Neu5Ac and the Neu5Acα(2,6)-Gal motif, is essential to advancing glycobiology. While α-sialylation remains one of the most difficult glycosidic linkages to generate chemically, the development of novel leaving and protecting groups in recent years has made their production less formidable. The Fukase lab utilized the high-throughput capabilities of microreactors to investigate several sialyl donors with diverse protecting group chemistries. Thus far, optimization studies have been performed with sialyl donors bearing *N*-Pthyalyl, azide, or *N*-acetamide at the C-5 position [108, 110, 111]. In each case, one of two microreactor systems was employed. The first system consisted of a commercial steel IMM micromixer compartment connected to two inlet tubes, through which reagent solutions were introduced, and a single outlet tube, through which the product solution was eluted [108, 110]. The second setup consisted of an integrated microfluidic/batch platform in which reagents were initially mixed inside of the micromixer system and then channeled into a flask for the reaction [111]. Tanaka and coworkers consistently observed a higher reactivity of sialyl donors using the microfluidic conditions than under batch conditions, a trend they attributed to the vigorous and rapid mixing achieved with the micromixer system. Decomposition of the sialyl donor and sialoside product is a persistent issue in chemical sialylation. Heat generated from slow mixing in flask reactions often causes degradation of the donor and product. Rapid heat transfer provided in microreactors mitigates hydrolysis of the sialyl donor, and the fine-tuning

of residence times allows sialoside products to be removed before they can degrade. These findings suggest that other convenient yet understudied glycosyl donors may warrant re-investigation for use in chemical glycosylation. In particular, their examination in microfluidics may help identify the factors affecting their reactivity or lack thereof.

β-Mannosylation is another challenging reaction required for synthesis of N-glycans. Stereoselective schemes have been established but often fail to scale effectively. For instance, a β-mannosylation protocol developed by the Fukase lab requires large quantities of the bulky activator tetrakis(pentafluorophenylborate) ($TMSB(C_6F_5)_4$), which is costly to obtain and impractical for high-volume productions [112]. While trimethylsilyl trifluoromethanesulfonate (TMSOTf) can serve as a cheaper alternative for large-scale synthesis, the acid was found to be sensitive to reaction volume and the addition speed of the acid in batch processes. Fukase and coworkers leveraged these observations to optimize β-mannosylation reactions in microreactors, where the speed of TMSOTf addition and reaction volume could be tightly controlled [109]. Here, the hybrid microfluidic/batch setup was used to synthesize a characteristic *N*-glycan motif, Manβ(1,4)-GlcNAc. While stereoselectivity was lower in the microfluidic/batch system, the β-mannoside product was obtained with a similar efficiency relative to the batch reaction. Notably, unlike the batch reaction, the yield and stereoselectivity of the microreaction remained consistent for a scaled-up synthesis in which the microfluidic/batch apparatus was continuously operated for an extended period.

Seeberger and colleagues have explored α-mannosylation workflows using continuous flow microreactors. For this work, installation of α(1,2)-linked mannoses was optimized inside of the aforementioned 5-port microreactor using a standard trichloroacetimidate (TCA) donor and two novel phosphate donors [98, 106]. In this case, the authors screened a broader range of reaction parameters, including reaction time, substrate concentration, temperature, and solvent. Optimal parameters for the TCA donor were applied to two larger-scale, continuous flow syntheses of the disaccharide, which achieved high yields of 81% and 93%. Additionally, when these optimized microreactions were converted to batch reactions, researchers detected nearly identical yields (98%, and 91%) for both reactions. Thus, not only can microfluidics perform comparably to traditional in vitro methods, but parameters optimized on-chip are transferable to batch processes.

Multi-step synthesis of oligosaccharides

Given the shorter reaction times they enable, microfluidic devices were immediately identified as tools to optimize and streamline multi-step assembly of complex oligosaccharides. As with batch systems, microfluidic protocols require tedious purification steps. Researchers have attempted to address this issue by equipping glycosyl donors with tags to enable solid-phase extraction of intermediates and products [18, 113]. Fukase and coworkers were the first to combine continuous flow microreactors with solid-phase chemistry to carry out high throughout, oligosaccharide synthesis [113]. To this end, researchers devised a novel podand-type ether tag to facilitate isolation of intermediates and products using a "synthesis based on affinity separation" (SAS) strategy. Glucosaminyl acceptors were equipped with the podand-type ether by direct conjugation or via a linker and then combined with glucosaminyl or galactosyl donors to generate disaccharide and trisaccharide structures. Automation of the reaction and purification occurred by constructing a microreactor comprised of a commercial IMM micromixer connected to a stainless steel tube reactor and column that served as the affinity separation unit. Using this approach, the authors demonstrated that SAS can improve the synthesis and purification of oligosaccharides, especially when coupled with microreactors to automate the process.

Carrel et al. subsequently reported the assembly of a homotetramer of protected β-1,6 linked D-Glc using the aforementioned 5-port microfluidic [18, 98]. Performing iterative glycosylation reactions presented additional purification demands, which the authors addressed using fluorous chemistry. Moreover, fluorous tags were added to the glycosyl acceptor to allow for isolation of target products by fluorous solid-phase extraction. Building on previous work, the protocol employed Fmoc-protected glycosyl phosphate donors [106]. Assembly of the di-, tri-, and tetrasaccharide products was optimized in the microfluidic, and peak performance was observed at room temperature for reaction times ranging from 20 to 60 s. Notably, yields of 90% or greater were achieved for each reaction step. This work provided the first example of integrating solid-phase extraction and fluorous chemistry within a microfluidic system designed for glycan synthesis.

More recently, Fukase and colleagues implemented an integrated one-pot and microflow procedure to effectively scale-up the synthesis of GlcNAc-containing trisaccharides, α-gal and H-antigen [114]. After scaling the one-pot procedure, synthesis of the disaccharide intermediate became prone to overreaction and reduced yields of the intermediate from 80 to 57%. By switching from a one-pot system to a continuous microflow device during scale-up, the reaction time was reduced from 5 to 1 min and product yield increased from 80% in the initial one-pot system to 94% in the scaled microflow procedure.

Synthesis of glycan precursors

Within the past decade, many groups have leveraged microfluidics to address additional challenging

transformations related to chemical glycosylation. Prominent examples include protecting group manipulation, peptide glycosylation, and the conjugation of carbohydrates to synthetic polymers [115–119]. Preparation of starting materials, such as the synthesis of glycosyl donors and/or acceptors, and post-reaction clean-up, such as the purification of final products, are often rate-limiting steps in glycan synthesis. Consequently, many researchers have employed microfluidics to execute these steps for glycosylation reactions that proceed efficiently under batch conditions. Microfluidics have garnered the most use for synthesizing glycosyl donors and acceptors and manipulating protecting groups on these substrates. Fukase and coworkers were the first to demonstrate this application for the reductive opening of 4,6-*O*-benzylidene acetals, a common reaction performed during protecting group manipulation of monosaccharides [120]. Because the reaction is exothermic, precise temperature control and efficient mixing of the reagents was required to prevent degradation of the benzylidene group. Control over both parameters was conveniently achieved using a microfluidic system. After screening for optimal reaction conditions, the authors were able to synthesize the target 6-*O*-benzyl derivative within the span of a minute with a final yield of 93%. The microflow protocol out-performed the corresponding batch procedure, which typically required an hour-long reaction time, more dilute concentrations of the acid, and only delivered 58% yield of the desired product. Similar results were obtained for 4,6-*O*-benzylidene acetals of galactose, glucose, and glucosamine.

In a subsequent study, Fukase and coworkers applied the same microreactor to the preparation of sialyl donors for the solid-supported assembly of N-linked oligosaccharides [121]. Both monosaccharide and disaccharide building blocks were synthesized on a 5–10-g scale, which enabled the solid-phase synthesis to be scaled under batch conditions. Kawakami et al. took a similar approach to carry out the multi-step protection of a fluorous-tagged glucose monomer [115]. The fluorous tag allowed integration of fluorous liquid phase extraction (FLPE) into the microreactor system to expedite the purification process, an often time-consuming step in carbohydrate synthesis. Synthesis was carried out in five steps beginning with the installation of the fluorous tag at the reducing end of the glucose precursor. The microreactor enabled each synthetic step to proceed at room temperature within 30 to 100 min, with the overall synthesis taking 6 h with an additional 3 h for the FLPE separation (9 h total). Although this method did not produce greater yields of intermediates and target products, it enabled each reaction step to be more efficiently optimized in comparison to the flask reactions.

Synthesis of glycoconjugates

Researchers have endeavored to adapt microfluidic platforms to the glycosylation of other biomolecules, particularly peptides [116, 118]. Only two attempts have been made thus far likely because of the complexity of peptide glycosylation. The difficulty stems from the low nucleophilicity of the amide nitrogen. Efficient strategies for N-linked glycosylation exist but rely on explosive solvents that make them unattractive for large-scale synthesis. Devising synthetic protocols wherein less hazardous solvents can be utilized to carry out high-yield peptide glycosylation is needed. Tanaka et al. developed such a strategy to prepare N-linked glycopeptide fragments using the aforementioned microfluidic/batch apparatus [116]. The model reaction consisted of monosaccharide or disaccharide sugar donors, an asparagine acceptor, and TMSOTf in three different solvent systems (nitromethane, dichloromethane, and propionitrile). The microfluidic optimized reactions provided yields of 81% and 84% with the monosaccharide and disaccharide donors, respectively, demonstrating the potential of microfluidics to improve glycoconjugate reaction efficiency and product yields.

Microreactors have also been developed to synthesize *O*-linked glycopeptides for various uses, including clinical applications. The overexpression of *O*-linked glycoproteins is a signature of adenocarcinomas, which has sparked interest in their use as potential cancer vaccines [122]. Mucin-1 (MUC1) is one such *O*-linked glycoprotein and fluorinated analogs of tumor-associated MUC1 glycans, such as the T_N antigen, have garnered attention for this purpose as they enhance the immunogenicity and stability of these molecules. Chemical preparations of fluorinated carbohydrate antigens suffer from the typical challenges observed in chemical glycosylation. Oberellig et al. addressed this issue by performing microfluidic-assisted screening of reaction conditions for the production of O-linked glycosyl amino acids using fluorinated sugars [118]. In the model reaction, fluorinated galactose was transferred onto T_N antigen (GalNAcβ-Threonine (Thr)) in the presence of TMSOTf to generate a fluorinated analog of the Thomsen-Friedenreich (T) antigen (Galβ(1–3)-GalNAcβ-Thr). The microfluidic reduced the reaction time from 12 h at room temperature to 90 min at 30 °C. While the batch reaction provided higher yields (89%) and stereoselectivity (α/β = 1:10), the microreactor still performed well (72% yield, α/β = 1:6) and its higher throughput capabilities allowed the authors to examine the impact of temperature on stereochemical outcomes. Furthermore, this procedure can serve as a template for preparing other O-linked carbohydrate antigens with therapeutic promise.

In addition to natural glycoconjugates, researchers have leveraged microfluidics to engineer biomimetic

glycoconjugates. Wojick et al. developed a microphotoreactor system to generate heteromultivalent glycopolymers comprised of a poly/oligo(amidoamine) (PAA) scaffold [119]. The microflow system enabled sequence-controlled and non-immunogenic conjugation of carbohydrates to amino acid building blocks by photochemical thiol-ene chemistry (TEC). These glycosyl amino acids could then be assembled into specific oligosaccharide structures via solid-phase synthesis. This microflow platform produced large quantities of glycosyl amino acids and streamlined the purification process, which was previously complicated by the use of copper-catalyzed conjugation strategies. Overall, the microflow system proved to be versatile as two complementary versions of the device were developed for the synthesis of homomultivalent glyco-PAAs and heteromultivalent glyco-PAAs.

Modeling glycosylation and advances towards automation

Although microfluidics often offers greater reaction efficiency in practice, this approach still requires optimization of each unique glycosylation reaction. Moreover, for each unique combination of acceptor, donor, and activator, the reaction parameters must be readjusted to retain adequate yields and stereoselectivity. Developing guidelines that can assist synthetic chemists in selecting appropriate reaction conditions without having to manually optimize every reaction step is highly desired. To do so will require a better understanding of the factors that influence stereochemistry and product yields during these chemical transformations. The Seeberger group has recently started to do this by conducting a systematic investigation of the permanent and environmental variables influencing the stereoselectivity of glycosylation reactions [107]. To do so, they developed and utilized a computer automated microreactor/HPLC platform modeled after the five port microfluidic reported in their previous work (Fig. 4) [98]. Using this system, the identity of the leaving group, donor substrate, and acceptor substrate were varied to examine the stereoselective preferences of different glycosylation partners. The influence of environmental conditions such as the reaction temperature, stoichiometric ratios of donor/acceptor pairings, choice of activator, the presence of water, and the solvent medium, were also investigated in this fashion. In total, 270 automated glycosylation experiments were performed from which the authors concluded that coupling partners (i.e., donor/acceptor pairing) express stereochemical preferences, which can be enhanced, diminished, or overridden by the choice of environmental conditions. Additionally, the degree of influence of each permanent and environmental factor investigated was quantified and compared. Overall, this study lays the groundwork for the utility of microfluidics in developing guidelines for the rational design of oligosaccharide syntheses.

Critical analysis

Microreactors have predominantly aided chemists in streamlining the parameter screens for challenging glycosylation reactions. Many groups have demonstrated high-yielding and stereoselective formation of historically formidable glycosidic linkages, such as α-sialylation and β-mannosylation. However, their adoption as a conventional tool for glycan synthesis remains slow. This is most apparent in their limited use for glycoside bond formations other than α-sialylation, β-glucosylation, and β-mannosylation. For the full potential of microfluidics to be realized, a broader spectrum of glycosidic linkages and monosaccharides must be incorporated into microfluidic protocols. In a similar vein, microreactors employed in carbohydrate chemistry lack variety in their overall design. As chemical glycosylation reactions are overwhelmingly performed with organic solvents, most devices are constructed out of silicon, glass, or steel. Consequently, device fabrication is typically more expensive and limited to labs with access to the requisite microfabrication equipment.

Despite their introduction to the field over two decades ago, few labs have leveraged microfluidics to perform total synthesis of oligosaccharides. Most applications have involved preparations of small oligosaccharides, such as di-, tri-, and tetrasaccharides, and, for complex targets, the precursor compounds are often oligosaccharide fragments chemically prepared in batch reactions. Several labs have used microfluidics to prepare sugar building blocks but rarely is the subsequent assembly of the oligosaccharide or glycoconjugate also carried out via microreactions. In general, procedures in which microreactors are used for the entire glycosylation process, including preparation of precursors, the glycan transfer reaction(s), and post-reaction processing remain to be seen. Integration of every step of glycan synthesis into continuous flow devices would facilitate the creation of fully automated chemical approaches for broader application of these techniques by non-specialists. The limited understanding of how different variables influence glycosylation has hindered the creation of such devices. Seeberger and colleagues are paving the way for developing the insight required for automating chemical synthesis on chip. The field will benefit from on-going efforts to identify and quantify the degree to which reaction and environmental variables influence the yield, stereoselectivity, and regiochemistry of glycosylation reactions [123]. It is also worth noting that the vast majority of microfluidic-assisted glycosylation protocols almost always use trichloroacetimidate donors and TMSOTf, an activator [98, 113]. Application of other activator and donor systems should be explored as well.

Microfluidic technologies for enzymatic glycan synthesis

As discussed in the "State-of-the-art methods for glycan synthesis" section, chemists have increasingly employed glycoenzymes to circumvent the complexities of chemical glycan synthesis. Nevertheless, the widespread use of enzymatic and chemo-enzymatic methods is still hindered by the high cost and low availability of donor substrates, as well as the limited number of recombinant glycoenzymes that are well-characterized. Furthermore, many glycoenzymes exhibit slow kinetics in batch synthesis which has undermined their use in preparative scale syntheses [124]. To address these issues, researchers have leveraged microfluidic platforms to streamline the optimization of enzymatic reactions, facilitate recycling of enzymes, and automate glyco-enzymatic synthesis [77]. Solid-phase methods have predominantly been used in these devices. Devices that use solution-phase methods are rare and will therefore not be discussed here [125, 126]. Two versions of solid-phase devices have been created (Fig. 3C). The first includes enzymatic microreactors with immobilized substrates, which allows easy isolation of glycan targets from enzymes and unused reagents via simple rinsing steps. The second includes microreactors that contain immobilized enzymes, which facilitates the recycling of precious biocatalysts and, in some cases, improves enzyme stability [127].

Immobilized enzyme microfluidics for enzymatic glycan synthesis

Enzyme immobilization is a mature technology that has been widely employed to carryout enzymatic synthesis of carbohydrates [85, 127–134]. Within the past two decades, immobilized enzyme technology has increasingly been combined with microfluidics to augment the performance of glycoenzymes. To this end, researchers have leveraged immobilized enzyme microreactors (IEMRs) containing phosphorylases and glycosyl hydrolases to develop novel immobilization techniques and support modifications to enhance the catalytic effectiveness of these biocatalysts. They have also leveraged similar systems to characterize the kinetics of glycoenzymes, which has been an elusive feat in traditional batch systems [60]. IEMRs containing Leloir GTases have largely been developed to facilitate multi-step enzymatic syntheses and to automate enzymatic glycosylation reactions. Below, these applications of glyco-catalytic IEMRs are expounded upon in more detail.

IEMRs containing phosphorylases and glucosidases

IEMRs containing GHs, such as β-glucosidases, and non-Leloir glycosyltransferase, such as sucrose phosphorylases (SPases) and cyclodextrin glycosyltransferases (CGTases), have primarily been used to characterize the kinetics of glycan synthesis using these immobilized enzymes. Indeed, the first glyco-catalytic IEMR was developed by Nidetzky and coworkers in 2007 for this purpose [135]. This microfluidic platform consisted of a PDMS-based multichannel reactor inside of which recombinant β-glucosidase from *Pyrococcus furiosus* was covalently immobilized using the 3-aminopropyl triethoxysilane(APTES)-glutaraldehyde method. The microchip was connected to a UV/Vis spectrophotometer to enable on-line monitoring of the enzymatic hydrolysis of lactose to glucose and galactose. Measurements of specific activity revealed that the immobilized enzyme retained 3% of the activity of the free enzymes in solution. A systematic analysis of the immobilized enzyme kinetics indicated that the tethering strategy caused this discrepancy in enzymatic activity. Covalent immobilization strategies, like the glutaraldehyde method, often proceed in a random fashion. This highlights the importance of developing oriented immobilization strategies to avoid adverse outcomes in activity.

The issue of orientation in enzyme immobilization has led several groups to develop novel tethering strategies using glyco-catalytic IEMRs. Tethering strategies are typically evaluated for their ability to achieve flow-resistant immobilization and high enzyme loadings, and minimize loss of specific activity in the immobilized protein. GHs, particularly prokaryotic β-glucosidases and β-galactosidases, are popular model systems for studying enzyme immobilization in microfluidics [135–138]. Nidetzky and coworkers developed a non-covalent immobilization method that allowed oriented adsorption of enzymes [139]. The strategy utilizes engineered enzyme chimeras, wherein the N- or C-terminal of the enzyme is fused to a polycationic peptide, Z_{basic2}, that can couple to anionic surfaces via charge complementarity. Thus, enzyme orientation is controlled by the location of the fusion peptide in the enzyme chimera. The group evaluated this methodology in a series of studies using glass and stainless steel microfluidics comprised of immobilized SPases [140–143]. They initially investigated the impact of multivalency on tethering strength and enzyme activity. They specifically compared "monovalent" enzymes, which contain a single Z_{basic2} fusion peptide, to "divalent" enzymes, which carry two fusion peptides or consist of monovalent constructs that form homodimers in solution (Fig. 5) [140]. Kinetic analysis indicated that SPases immobilized via Z_{basic2} retained the intrinsic activity of the free enzymes. Additionally, the multivalent constructs outperformed the monovalent constructs in anchoring and operational stability. The group subsequently compared the Z_{basic2} strategy to the covalent fixation of His-tagged SPase, and found that both immobilization strategies exhibited similar levels of enzyme loading, catalytic activity, enzyme retention, and operational stability inside of the microchannel [142]. Given that Z_{basic2} is easier to implement as a one-step and reversible

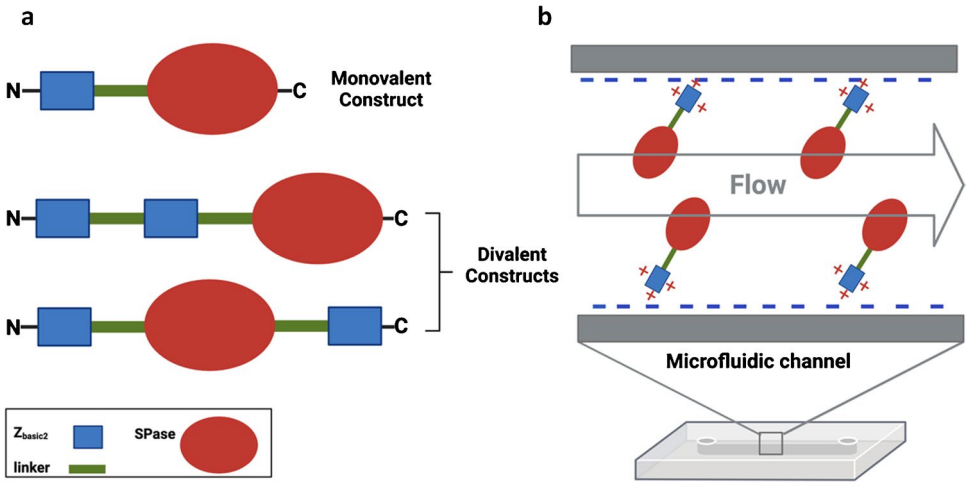


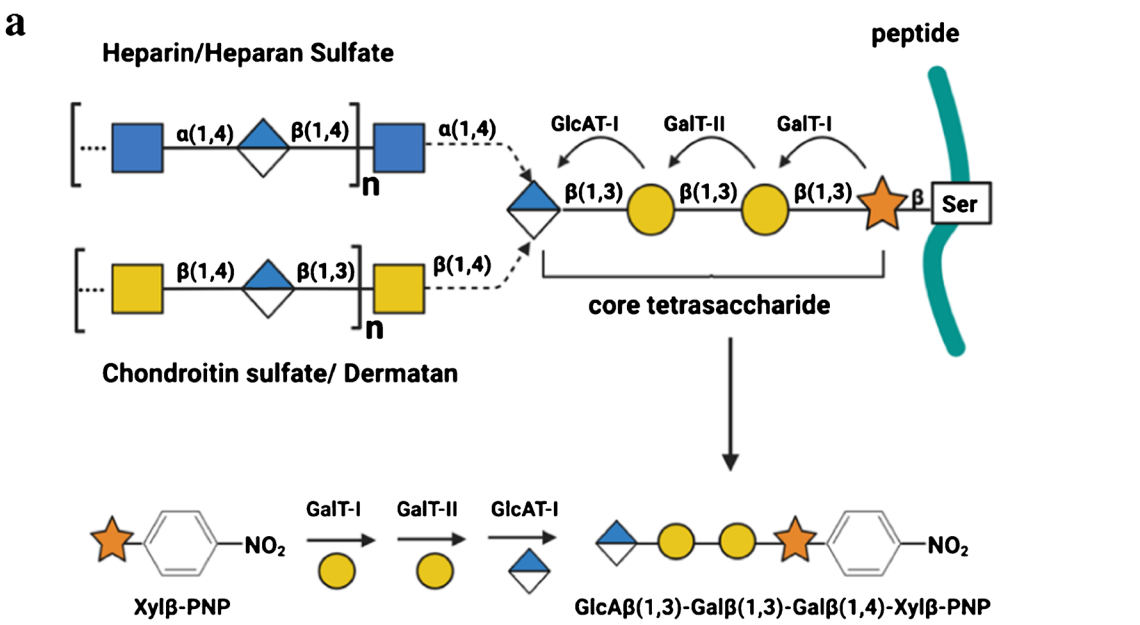
Fig. 5 A diagram explaining the concept of the Z_{basic2} immobilization method [140]. (**a**) SPases (red oval) were engineered to contain N-terminal or C-terminal fusions to a polycationic peptide, Z_{basic2}, (blue box) via a linker (green line). Monovalent constructs were prepared by adding a single fusion peptide to SPases. Divalent constructs were prepared by adding two N-terminal fusion peptides or both a N- and C-terminal fusion peptide to the enzyme. (**b**) The enzyme chimeras from (**a**) were non-covalently immobilized inside of a microfluidic channel by charge complementarity between the anionic surface of the channel and the Z_{basic2} peptide (only monovalent constructs shown). This allows enzymes to tether to the channel in an oriented manner. Adapted from reference 141 with permission from John Wiley and Sons. Created with BioRender.com

immobilization process, this strategy is preferable for use in IEMRs if functional chimeras can be generated. In general, the technique is well-suited for workflows that involve enzymes with intrinsically low specific activities.

Aside from protein orientation, the performance of IEMRs is also heavily influenced by the amount of surface area accessible to enzymes. IEMRs must be designed to accommodate a sufficient quantity of active enzymes. Generic microchannels that lack packing or internal structures offer limited surface area for enzyme functionalization and these features often cause issues with back-pressure that can complicate device fabrication [144, 145]. Modifying the internal surfaces of IEMRs is often needed to boost enzyme immobilization. Nidetzky and colleagues increased the loading of SPase inside of a glass microreactor by coating the internal walls with porous silica nanosprings [141]. These microstructures have a large specific surface, exhibit low resistance to fluid flow, and present silanol groups, which allowed enzymes to be tethered to the surface using the Z_{basic2} method. The nanosprings significantly increased the enzyme loading capacity of the flow cell, enzyme resistance to surface detachment, as well as the conversion of sucrose and potassium phosphate to phosphorylated glucose. More recently, zinc-oxide nanowires were grown on the inner walls of a glass microtube to enhance the activity of covalently tethered *Thermotoga maritima* β-glucosidase [146]. While less preferred, monolithic microreactors and hybrid platforms that incorporate both packed bead and open tubular sub-compartments have also been developed to enhance biocatalysis of glycoenzyme-containing IEMRs [147, 148].

IEMRs containing Leloir glycosyltransferases

Glyco-catalytic IEMRs have allowed chemists to streamline and, in some instances, automate multi-enzyme cascades that involve Leloir GTases. Multi-enzyme reactions are a hallmark of enzymatic and chemo-enzymatic workflows for glycan synthesis. Unlike chemical processes, enzymatic glycosylation has yet to be fully automated. IEMRs are ideal platforms for automating enzymatic glycan synthesis as they can readily integrate multiple processing units in series or in parallel. This modularity affords spatio-temporal control over reactions, as enzymes can be compartmentalized to separate competing reactions and mitigate cross-inhibition, or colocalized to enhance the transport of substrates between biocatalysts [99]. Several groups have designed compartmented IEMRs to carry out multi-step synthesis of glycosides. The first demonstration was reported by Ono et al. who devised a platform for the three-step synthesis of a core tetrasaccharide (GlcAβ(1,3)- Galβ(1,3)-Galβ(1,4)-βXyl) that serves as a universal precursor in GAG biosynthesis (Fig. 6a) [149]. Three sequential glycosyltransferase reactions were carried out to convert PNP-βXyl to the target tetrasaccharide. Each GTase contained a FLAG fusion peptide for immobilization on agarose beads. The microreactors consisted of a single microchannel partitioned into three chambers to house each enzyme separately, allowing chamber conditions to be tailored to each enzyme (Fig. 6b). The system was equipped with a temperature control and multiplexed with LC/MS to enable in line monitoring of product formation. Using this setup, the issue of feedback


a
Heparin/Heparan Sulfate
peptide
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GlcAT-I
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Ser
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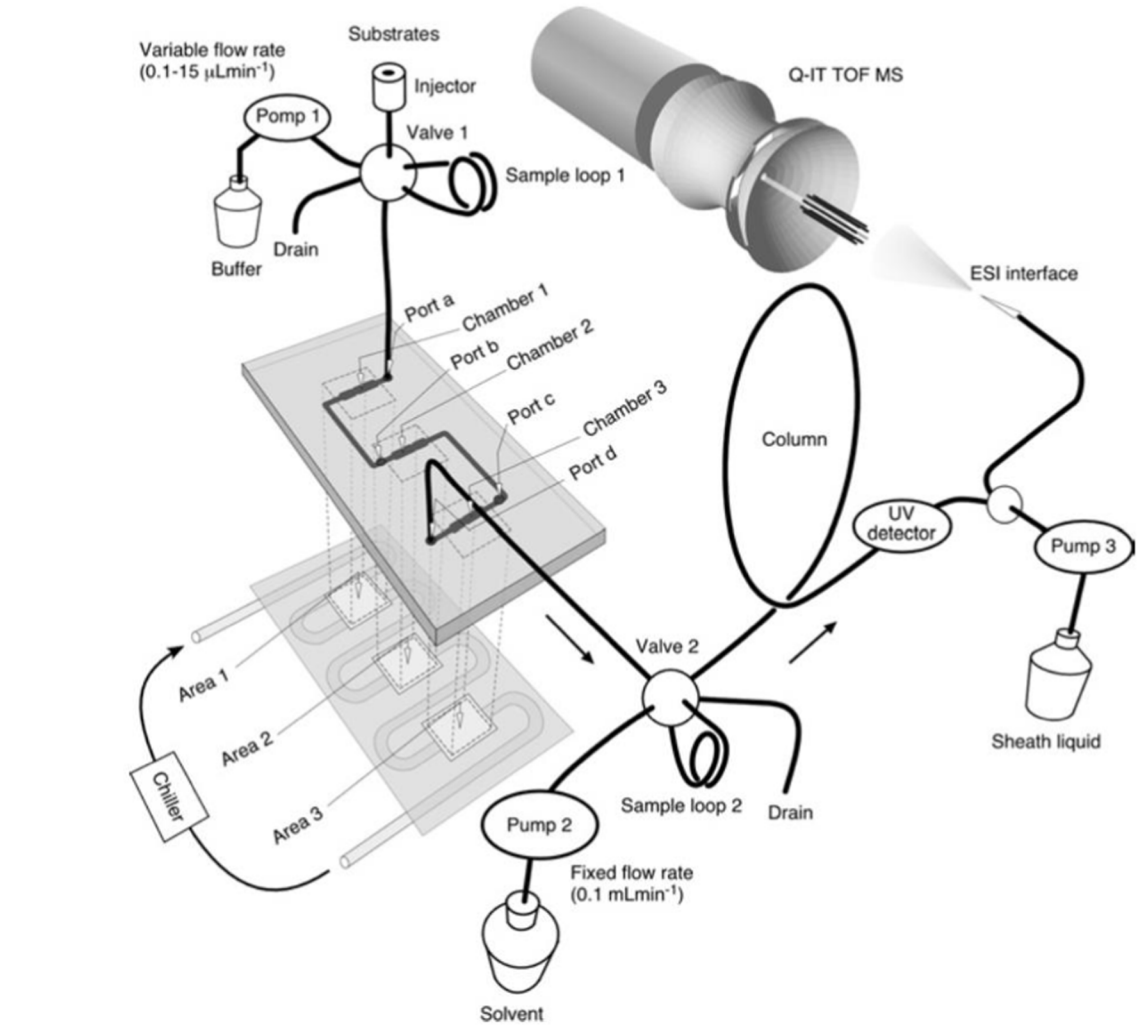
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Variable flow rate
(0.1-15 μLmin-1)
Substrates
Injector
Pomp 1
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Port a
Chamber 1
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Chamber 2
Port c
Chamber 3
Port d
Column
UV
detector
Pump 3
Sheath liquid
Area 1
Area 2
Area 3
Chiller
Valve 2
Sample loop 2
Drain
Pump 2
Fixed flow rate
(0.1 mLmin-1)
Solvent

◂**Fig. 6** Example of a compartmented microfluidic system designed for enzymatic glycosylation reactions. (**a**) An illustration of the structure of the target tetrasaccharide and the reaction scheme for the three-step conversion of PNP-βXyl to the tetrasaccharide (βGlcA(1,3)-βGal(1,3)-Galβ(1,4)- βXyl-PNP), which is catalyzed by GalTI, GalTII, and GlcAT-I [149]. (**a**) A schematic of the multi-chamber microfluidic designed by Ono et al. for the multi-step synthesis of a core tetrasaccharide unit found in GAGs. Reproduced and adapted from reference 150 from the Royal Society of Chemistry. Panel **b** was created with BioRender.com

inhibition was averted. Additionally, the authors were able to characterize the kinetics of the *Drosophila melanogaster* β-1,4-galactosyltransferase I (β-1,4-GalT) which was the first report of its kind for this enzyme. Most notably, the localization of reaction steps within distinct compartments mimics the organization of the Golgi apparatus and suggests that the platform could serve as a template for investigating the regulatory mechanisms of the Golgi ex vivo.

Over a decade later, Franzreb and coworkers developed a compartmented flow microreactor system (CFMS) to automate enzymatic glycosylation reactions [124, 150]. The platform accommodated an analog and digital input/output module, a series of reaction modules designed for either short- or long-term reactions, and an integrated spectrometer module to enable online analysis [151]. All modules were interlinked and controlled by an automation software. Enzymes were immobilized on nickel-functionalized magnetic microcarriers via poly-histidine tags. The updated prototype was initially used to screen reaction conditions to produce the disaccharide, N-acetylactosamine (LacNAc) using immobilized β-1,4-GalT. The automated setup enabled contactless assessment of various reaction conditions, which streamlined kinetic analysis and minimized product loss during spectrophotometric analysis. The latter was achieved because the carrier-bound enzymes were easily isolated from the reaction solution by placing a magnetic array next to the tube. After spectrophotometric analysis, the reaction solution could be reunited with the carrier-bound enzymes for further processing. This contrasts with liquid handling steps required for off-line analyses, where serial pipetting often results in both product and enzyme loss. When applied to continuous production of LacNAc, the microflow device yielded preparative amounts of the product within 8 h.

Franzreb and colleagues subsequently applied their automated CFMS to a multi-step synthesis of the non-sulfated, human natural killer cell 1(HNK-1) glycan epitope [124]. Here, the CFMS incorporated six magnetic bead–bound enzymes which collectively synthesized two donor substrates, UDP-Gal and UDP-GlcA, and the two-step conversion of GlcNAc to HNK-1 catalyzed by β-1,4-GalT and β-1,3-GlcAT. As such, the enzymes were compartmentalized into four separate reaction modules. Optimal parameters for each module were determined in the device using a "one-factor-at-a-time" approach and were found to correlate well with optimal conditions reported for the free enzymes. The CFMS device provided a high yield of 96% HNK1, a 40% improvement over the yield obtained using free enzymes in solution under the same condition. This discrepancy reinforces the importance of spatio-temporal organization in enzymatic synthesis.

In some cases, researchers have exploited enzyme colocalization to improve product yield. Nidetzky and coworkers developed a packed-bed microreactor comprised of Leloir GTases to enhance the production of a natural product, nothofagin [152]. The reactor was fabricated using a repurposed FPLC column packed with negatively charged polymethacrylate particles to allow oriented enzyme immobilization with Z_{basic2} fusion peptides. The reaction sequence involved synthesis of the donor substrate, UDP-Glc, catalyzed by sucrose synthase from soybean and the subsequent transfer of Glc onto the flavonoid, phloretin, catalyzed by *C*-glycosyltransferase from rice to generated nothofagin. Systematic analysis of each reaction step revealed that the enzymes were most effective when colocalized in the same chamber rather than isolated in separate chambers aligned in sequence.

More recently, Obst et al. also leveraged compartmentalized microfluidics to carry out the trienzymatic synthesis of CMP-Neu5Ac [153]. In vitro synthesis of CMP-Neu5Ac is confounded by cross-inhibition as pyruvate, a substrate in the second reaction step, inhibits the activity of CMP-sialic acid synthetase (CSS) in the third reaction step. The authors developed two configurations of a multi-chamber device to mitigate substrate inhibition. The first consisted of a non-compartmentalized format, wherein the three enzymes were co-immobilized within hydrogel dots placed inside both chambers. The second consisted of an analogous compartmentalized format, in which the other enzymes were confined to the first chamber and CSS was confined to the second in order to limit its exposure to pyruvate. Continuous production of CMP-Neu5Ac was successfully demonstrated in both the compartmentalized and non-compartmentalized designs. Relative to reaction cascades performed in bulk hydrogel systems, the microfluidic reaction significantly reduced the reaction time from 2 days to 20 min. The hydrogel scaffold imparted protective effects on the enzymes by hindering diffusion of inhibitory substances. Interestingly, both the non-compartmentalized and compartmentalized devices performed comparably to one another. This was attributed to the favorable effects of the reduced cross-inhibition conferred by the compartmentalization and to the shortened diffusion lengths between substrates and biocatalysts in the non-compartmentalized device.

Immobilized substrate microfluidics for enzymatic glycan synthesis

Microreactors containing immobilized substrates (ISMR) have also been explored for applications in enzymatic glycosylation. In these platforms, a glycan or aglycone substrate is tethered to a soluble or insoluble support material while enzymes in solution diffuse to the substrate to initiate the reaction. Several groups have used enzymatic synthesis to prepare immobilized glycan platforms [154]. These platforms are primarily configured as carbohydrate microarrays, wherein sugar precursors are covalently linked to glass, gold, or polymer surfaces via their reducing terminus and elaborated upon by the action of glycoenzymes dissolved in solution. Diverse glycans and aglycone substrates have been featured in microarrays including oligosaccharides, peptides, proteins, and lipids [76, 155]. The reactions typically proceed under batch conditions with glycoenzymes introduced in either a stepwise fashion or in a combinatorial manner akin to OPME synthesis. Despite the benefits they afford in terms of automation and isolation of intermediates, substrate-immobilized platforms generally exhibit slower reaction rates and lower yields relative to solution-phase systems.

A few researchers have leveraged microfluidics to increase the biocatalytic efficiency of immobilized glycan platforms. In a seminal work, Martin et al. carried out enzymatic sulfation of immobilized heparan sulfate using a digital microfluidic chip modeled after the Golgi apparatus [156]. This glass microfluidic consisted of an array of electrodes that enabled droplet movement to be directed by application of electric pulses. One droplet containing the substrate, heparan sulfate (HS), immobilized on magnetic nanoparticles, and another containing the enzyme, D-glucosaminyl 3-*O*-sulfotransferase isoform-1 (3-OST-1) and the sulfate donor, were directed to mix and then incubated on chip for the duration of the reaction (Fig. 7). Production of modified HS was assayed by binding of antithrombin coagulant III, which displayed higher affinity for the enzymatically treated sample. Thus, immobilized HS was successfully modified on-chip. This work marked the first demonstration of the enzymatic modification of an immobilized substrate in a digital microfluidic and was proposed as a first step towards realizing an artificial Golgi.

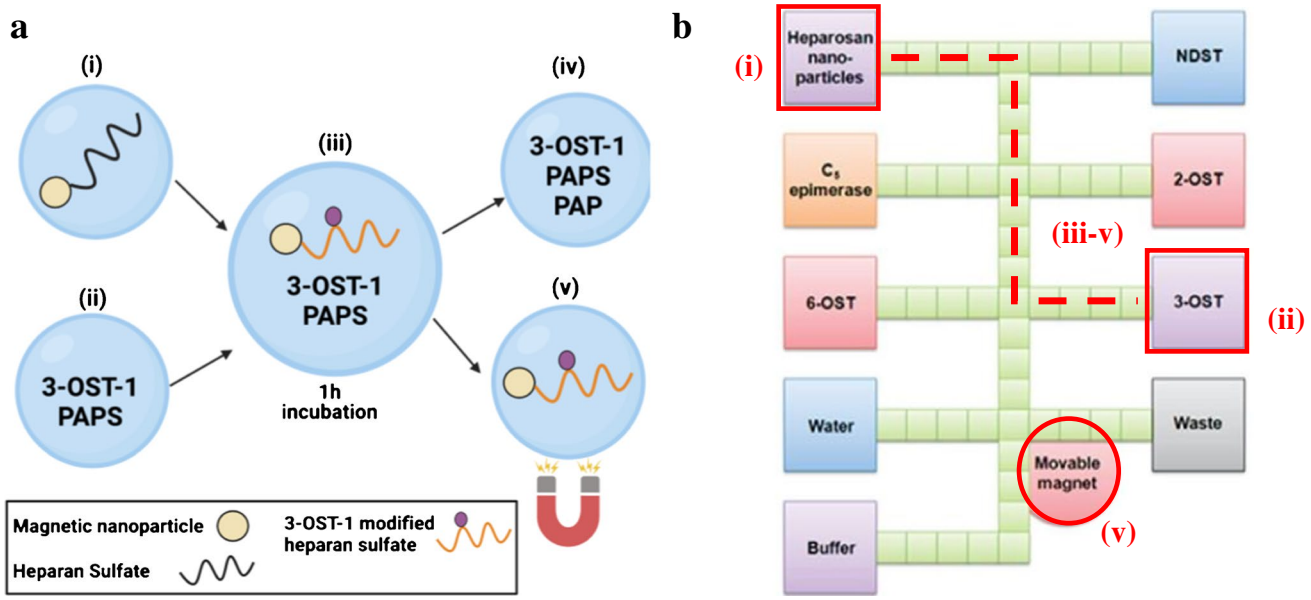

Fig. 7 An illustration of the reaction carried out in the Golgi-mimetic digital microfluidic developed by Linhardt and colleagues [156]. (**a**) A diagram of the on-chip modification of heparan sulfate by 3-OST-1. A droplet containing the 3-OST-1 and the phosphate donor, PAPS, (i) and a droplet containing the substrate, HS, immobilized on a magnetic nanoparticle (ii) were mixed and incubated on-chip (iv) to generate modified HS. A magnet was used to isolate a droplet containing modified HS (v) from the enzymes and excess reagents (iv). (**b**) An illustration of the Golgi-mimetic digital microfluidic used for the biosynthetic modification of HS. The large squares (multi-colored) are reservoir electrodes designed to hold reagents and enzymes. The components used for the droplet reaction depicted in (**a**) are outlined in red. Droplets (i) and (ii) were derived from the "Heparosan nanoparticles" and "3-OST" reservoirs respectively and joined to form droplet (iii) at a mixing site between the two reservoirs (red dashed line). After the reaction, the movable magnet (bottom right) was used to isolate modified HS (v). Adapted from reference 157 with permission from the American Chemical Society. Panel **a** was created with BioRender.com

Critical analysis

Several issues must be addressed before glyco-catalytic IEMRs can achieve widespread use. Foremost is the inclusion of aglycone and glycoconjugate acceptors in microfluidic-based syntheses, which has yet to be demonstrated. Additionally, a select few materials have primarily been used to construct glyco-catalytic IEMRs, including glass-based or stainless steel platforms. Incorporating less expensive materials for microreactor construction might aid in broader use of these platforms, particularly by labs lacking access to the specialized equipment required for microfabrication with glass and steel. The field appears biased towards the application of IEMRs. ISMR devices remain underexplored for enzymatic glycan synthesis. The lack of interest in these systems may stem from slow kinetics of glycosyltransferases acting on immobilized substrates. However, the ability to readily tailor the surface environment to incorporate diverse substrates, including aglycone precursors like proteins and lipids, warrants further investigation of these systems for use in enzymatic glycan and glycoconjugate production. Lastly, questions remain about whether microreactors always enhance the productivity of biocatalytic processes and whether optimal conditions identified for immobilized enzymes can be applied for optimal results in batch systems. In some cases, it may be ideal to scale biocatalytic processes using batch systems after screening for optimal parameters with a microreactor. More work is needed to understand when microreactor conditions are transferable to the analogous batch reactions.

Conclusion and future outlook

This review examined papers published over the past two decades on microfluidic devices used for glycan synthesis. We have focused on two categories of devices; those employed in chemical glycosylation reactions and those employed in enzymatic glycosylation reactions. When applied to chemical glycan synthesis, microfluidic devices facilitate the fast optimization of glycosylation reactions, which is a tedious process in traditional batch systems. When applied to enzymatic synthesis, microfluidics have primarily been designed as IEMRs to demonstrate better immobilization strategies that improve the activity of immobilized enzymes. Microfluidic technology has also facilitated multi-step enzymatic reaction cascades and enabled automation of enzymatic glycan syntheses. For both chemical and enzymatic syntheses, microfluidics consistently provide preparative-scale quantities of glycosides, making them suitable tools for preparing analytical glycan standards.

Nevertheless, the use of microfluidics for glycan synthesis is still in the development phase. Thus far, most microfluidic studies have primarily served as proof-of-concept platforms. These devices have yet to be widely adopted for chemical or enzymatic transformations. One reason for this is the reliance on construction materials, particularly glass and stainless steel that require access to and familiarity with specialized machinery. Additionally, despite their ability to streamline reaction optimization, microfluidic devices have yet to be applied to the high-throughput synthesis of glycan libraries or glycoconjugates for medical research. Indeed, no examples of this transition from prototyping to manufacturing were found in the literature. Synthesis must be reproducible within and across devices, and few studies have demonstrated the latter. This is especially a concern for IEMRs, where inconsistencies in support material or enzyme sample can significantly impact the immobilization efficiency and lead to variable reactor performance across different devices modeled after the same design.

Future work should prioritize the transition from prototyping to actual implementation in glycan functional studies. Enzymatic syntheses show the most promise in this regard, as they are typically performed in aqueous solutions and are compatible with PDMS devices, which are cheaper and faster to fabricate [157]. More studies should target the synthesis of complex glycoconjugates, namely glycoproteins and glycolipids, which remain underexplored. The ability to integrate supported lipid bilayer (SLB) technology with microfluidics could facilitate glycosylation reactions involving hydrophobic aglycones or transmembrane glycoenzymes. Our lab has successfully embedded functional membrane proteins in SLBs, and we have recently developed a multi-chamber microfluidic for the cell-free expression, glycosylation, and purification of green fluorescent protein (GFP). In the latter system, glycosylation of GFP was conducted with a bead-immobilized oligosacchyltransferase from *Campylobacter jejuni* using lipid-linked oligosaccharide donors [158, 159]. Finally, both IEMR and ISMR devices show promise as robust tools for kinetic and functional assays of glycoenzymes. Such platforms could help to resolve the activities of putative GTases in the CAZy database, which comprise the majority of entries, and facilitate the discovery of GTases with previously unidentified substrate specificities [160, 161].

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Declarations

Conflict of interest The authors declare no competing interests.

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