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Automated solution-phase multiplicative synthesis of complex glycans up to a 1,080-mer

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Carbohydrates play essential roles in nature, such as in cell–cell communication, cell growth and immunoresponse. However, the synthesis of structurally well-defined carbohydrates, especially large-sized glycans, is a challenging task. Here we report an automated solution-phase multiplicative synthesis of complex glycans enabled by preactivation-based, multicomponent, one-pot glycosylation and continuous multiplying amplification. This was achieved by making a dual-mode automated solution-phase glycan synthesizer. Using this synthesizer, a library of oligosaccharides covering various glycoforms and glycosidic linkages was assembled rapidly, either in a general promoter-activation mode or in a light-induced-activation mode. The automated synthesis of a fully protected fondaparinux pentasaccharide (an anticoagulant) was realized on the gram scale. Furthermore, automated ten-component tandem reactions were performed, allowing the assembly of arabinans up to a 1,080-mer using this automated multiplicative synthesis strategy.

Carbohydrates are ubiquitous in nature, and play significant roles in almost all life processes, such as cell–cell communication, cell growth and proliferation, pathogen–host interaction, and immunoresponse[1]. However, due to the intrinsic complexity of carbohydrate structures, furnishing pure and structurally well-defined glycans to study their functions is a formidable task. It usually involves multistep manual operations and requires a highly skilled workforce, making it time-consuming and laborious[2–4]. The development of synthetic technology has enabled automated synthesis, ranging from small pharmaceutical molecules to macromolecules such as nucleic acids or proteins[5–7]. Although automated solid-phase chemical synthesis[8,9] has been developed to assemble diverse glycans[10], this protocol requires an excess amount of building blocks at each coupling step[11], the synthesis cannot be scaled up and usually the reaction cannot be directly monitored[12], thus limiting its application to some extent. Although automated enzyme-mediated glycan synthesis has been reported[13,14], the enzymatic protocol is restricted by the source of enzymes and the scope of carbohydrate substrates. In addition, a few semiautomated platforms based on high-performance liquid chromatography (HPLC)-assisted automated synthesis[15], fluorous-tag-assisted automated solution-phase synthesis[16] and automated electrochemical solution-phase assembly[17] have also been established, but their synthetic applications are limited and the size of the constructed glycans does not exceed that of hexasaccharide. As a result, automated glycan synthesis is still in its infancy; a major bottleneck exists due to the absence of a robust, widely applicable protocol to access the requisite glycan chains[18], especially for the most commonly used solution-phase synthesis in the laboratory. Here we report a universal and highly efficient automated solution-phase synthesizer that is based on a preactivation one-pot multicomponent and continuous multiplicative synthesis strategy, averting the shortage of current approaches to extend the glycan size one by one (Fig. 1). By using this machine, the automated synthesis of a

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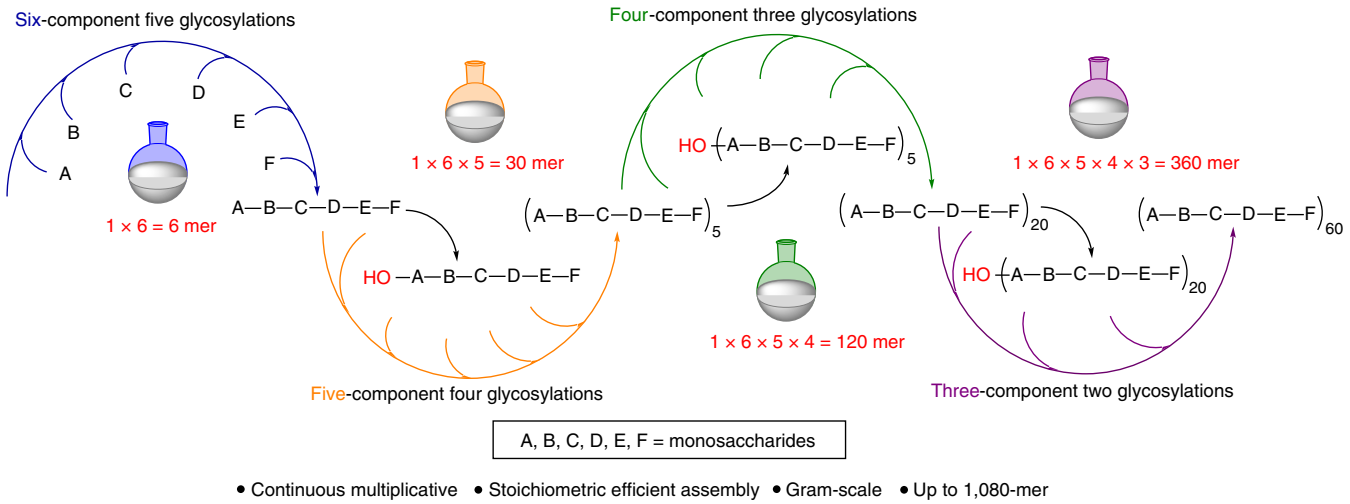

Fig. 1 | Automated glycan synthesis by a preactivation-based, one-pot, multicomponent and continuous multiplicative synthesis strategy. AMS not only enables the one-pot, multicomponent synthesis of oligosaccharides by a stepwise addition approach but can also be used to achieve the continuous multiplicative synthesis of polysaccharides by a rapidly multiplying amplification process. As illustrated, a 360-mer polysaccharide can be accomplished via four multiplying amplification steps ($1 \times 6 \times 5 \times 4 \times 3 = 360$ mer) using 14 glycosylations ($5 + 4 + 3 + 2 = 14$).

variety of oligosaccharides was realized either in a general mode or in a light-mediated mode. Importantly, the pentasaccharide precursor of the anticoagulant fondaparinux, a typical oligosaccharide drug, was efficiently assembled on the gram scale. Furthermore, the automated multiplicative synthesis (AMS) of complex polysaccharides up to a surprising 1,080-mer was achieved, taking glycan synthesis far beyond the synthesis of nucleic acids (up to 200-mer)[19] and proteins (up to 472-mer)[20].

Results and discussion

Automated solution-phase synthesizer

In 2004, we developed a preactivation-based one-pot glycosylation strategy[21], enabling multiple sequential glycosylations in a single reaction vessel. This protocol is independent of glycosyl donor reactivity, and different from programmable and hierarchical one-pot synthesis[22,23]. Because this approach does not require complex protecting-group manipulations and isolation of intermediates, it holds the potential for automated solution-phase synthesis (Fig. 2a). In this way, a range of glycans have been synthesized manually[24], including the arabinogalactan 92-mer[25]. In terms of green and sustainable chemistry[26], the preactivation-based one-pot glycosylation strategy can also be applied to light-driven glycosylation reactions[27], where either ultraviolet or visible light can be used for the activation of glycosyl donors. The two activation modes of this preactivation protocol means that several technical challenges need to be addressed to make the synthesizer: first, different stored homogeneous solutions must be delivered independently and in accurately measured amounts to the reactor in a closed system to avoid cross-contamination using minimal equipment; second, automatic sampling and injecting to achieve online monitoring; and third, all the operations have to be programmed and controlled.

The automated solution-phase synthesizer consists of three parts: a synthesis system, including a synthetic auxiliary system and an automatic injection system (Fig. 2b); an online monitoring system; and a programmable logic controller (PLC) (Fig. 2c). The development of the synthetic auxiliary system started from the design of a three-jacketed temperature-controllable reactor (25 or 100 ml) that can meet the requirements of both general promoter-mediated reactions and light-induced reactions. This reactor is conveniently equipped with a magnetic stirrer and connected to a cryostat with a temperature range adjustable between −80 and 100 °C, depending on whether choosing to be controlled by top-down illumination by a concentrated beam from a mercury lamp. The reactor has a sample inlet, an air outlet connected to an oil bubbler and a sampling port that is used to transfer the reaction solution to an online HPLC for reaction analysis and feedback. The sample inlet of the reactor is coupled to the automatic injection system, including building-block-delivery, reagent-delivery and solvent-delivery sections (up to 18 different components in total). When the injection volume is less than or equal to 1 ml, a 1 ml syringe pump provides power to withdraw the desired component via the corresponding channel of a ten-way valve and infuse it accurately into the reactor via a mass flow controller (MFC). When the injection volume is greater than 1 ml, a 10 ml syringe pump performs the same action to ensure the accuracy of all injection volumes; cooperation between the syringe pump and the MFC is achieved with a self-correcting algorithm, controlling the injection error to within 20 µl. This automatic injection system, which is closed and pressurized using inert gas supplied by two pressure regulators, also cleans the line with fresh solvent for the next injection, ensuring there is no cross-contamination between injections.

The online monitoring system, which is independent of the automated synthesis system, contains a controlled HPLC and a device for automatic puncture sampling and injection. It is a nearly real-time, direct and universal system that can make multiple consecutive non-crossover automatic sampling, filtering, injecting, analysis and comparison judgements to decide whether to continue automation or not. This system makes full use of solution-phase online detection capabilities to enable analysis and feedback of the reaction process (see Supplementary Information, section 1.2 for more details).

All the hardware communicates with the PLC which is controlled by upper computer software (Ye Glycosoft), which is written in LabVIEW and relies on a hierarchy of ten layers within an accessible graphical user interface (see Supplementary Information, section 1.1.5 for more details). Users can log in and enter the software interface to control the whole synthesizer, including but not limited to inputting reagent information, writing and performing a single action or combination of instructions, writing an automation program to open the automation operation, observing real-time dynamic images, recording and saving the data, and maintaining the various commercial hardware components. The control software is practical and easy-to-use to ensure stable operation of the synthesizer. By coupling the automatic synthesis system, the automatic monitoring system and the software control

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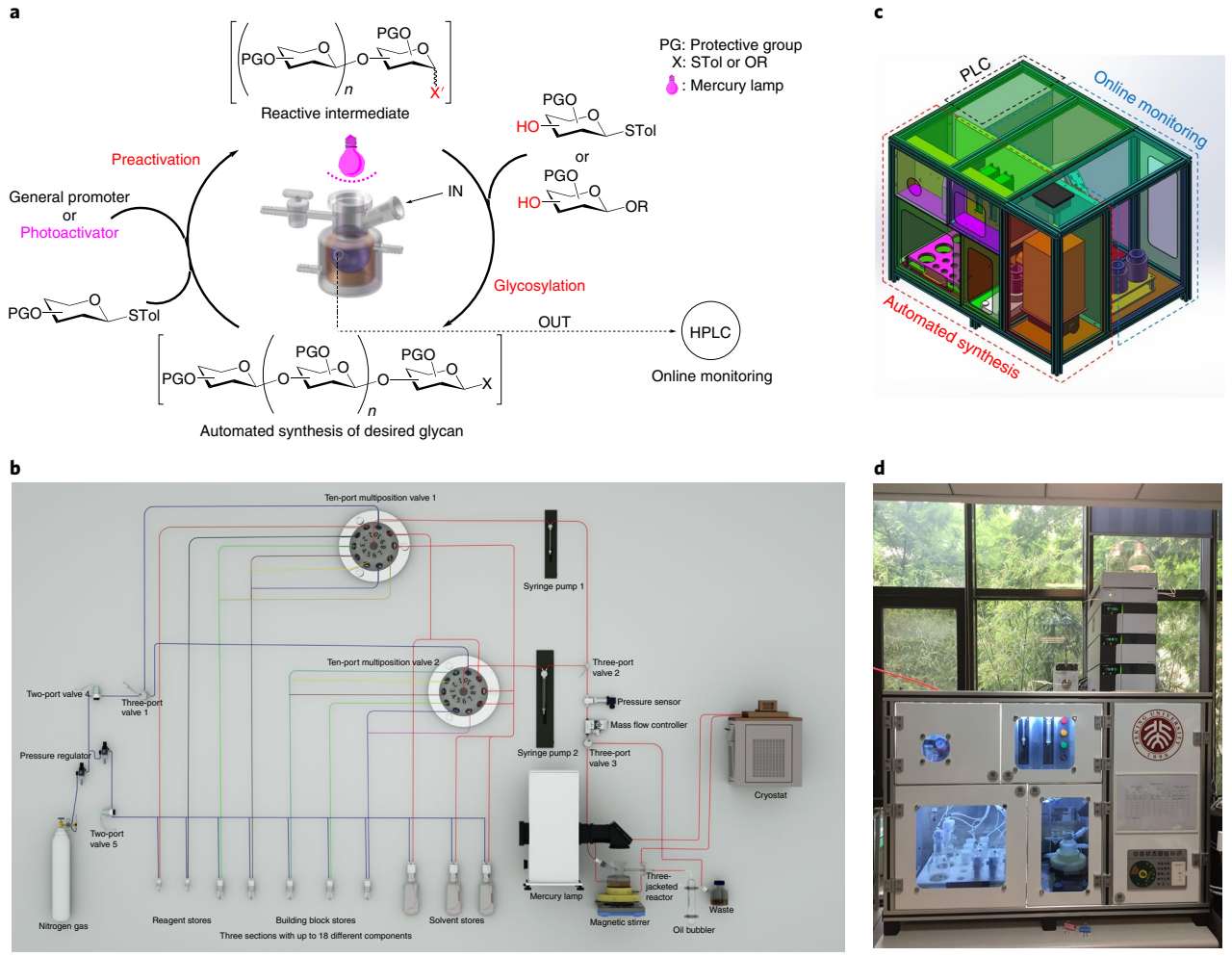

Fig. 2 | The solution-phase synthesizer. a, The principle of preactivation-based, one-pot glycan synthesis in general or light-induced modes. **b**, The hardware and flow diagram of the automatic synthesis system. **c**, The synthesizer consists of three parts: an automatic synthesis system, an online monitoring system and a PLC. **d**, The automated synthesizer. The housing dimensions are 0.8 m height, 1.2 m width, 0.9 m depth. Tol, *p*-methylphenyl.

system, a practical, safe and compact dual-mode automated solution-phase synthesizer has been assembled successfully (Fig. 2d).

Automated synthesis of various oligosaccharides in dual-mode

We have applied this automated solution-phase glycan synthesizer without issue to the synthesis of many biologically important oligosaccharides in dual-mode. Initially, in its general promoter-activation mode (Fig. 3a,b), the synthesizer was tested to rapidly obtain the model tetrasaccharide **8** starting from readily available monosaccharide building blocks (see Supplementary Information, section 2.2.1 for more details) by the four-component glycosylation reaction using Ph_2SO/Tf_2O as a general promoter. Subsequently, two β-1,6-linked pentaglucosides **4** and **5** were assembled from stoichiometric amounts of five monosaccharide components (1 × 5 = 5) in 56% and 54% isolated yields, respectively, which are better yields than those of manual one-pot synthesis[2], illustrating the usefulness of this new machine. Furthermore, after manual removal of the temporary levulinoyl (Lev) protecting group in pentasaccharide **5** to obtain glycosyl acceptor **6**, AMS was used to complete the automated synthesis of pentadecasaccharide **7** in 48% isolated yield by three-component iterative glycosylation (5 × 3 = 15), verifying the feasibility of the AMS protocol. In addition, the synthesis of pentamannoside **9** (1 × 5 = 5, 58% yield) was achieved. Tetrasaccharide **10** (1 × 4 = 4, 58% yield) and pentasaccharide **11** (1 × 5 = 5, 33% yield), composed of β-1,4-glucosamine and with diverse biological functions, were also assembled by the synthesizer from monosaccharides, in yields that were better than those obtained by automated electrochemical synthesis (28% yield for the tetrasaccharide)[28]. Globo H is a tumour-associated carbohydrate antigen that has been used in clinical trials for the development of carbohydrate-based anticancer vaccines[29]. In spite of the complexity of the Globo H antigen structure, which contains the challenging Gal-α-(1-4)-Gal linkage, the protected hexasaccharide **12** was obtained using a [1 + 2 + 1 + 2] strategy by iterative glycosylation in an automated solution-phase synthesis manner. Fucosyl GM1 (Fuc-GM1) is a sialic-acid-containing antigenic epitope that is found specifically in the tumour tissue of small-cell lung cancer and could be a good target for vaccine development[30]. The protected Fuc-GM1 **13** was furnished by iterative glycosylation in this synthesizer using a [1 + 2 + 3] strategy. Likewise, the protected A-antigen hexasaccharide **14** was also afforded smoothly.

Next, in light-induced activation mode using Umemoto's reagent as photoactivator, the model tetrasaccharide **8** was also achieved by four-component glycosylation (see Supplementary Information, section 2.2.2 for more details), indicating that the synthesizer can be applied to light-driven reactions. Based on the photoinduced one-pot [2 + 2 + 2] glycosylation to assemble a polyLacNAc hexasaccharide backbone[31] that exists in various *N*-linked glycans and tumour-associated carbohydrate antigens[23], the protected polyLacNAc octasaccharide **18** was assembled successfully via four-component multiplicative synthesis (2 × 4 = 8) in an automated photochemical way (Fig. 4). Two isomeric tetrasaccharides, lacto-*N*-tetraose and

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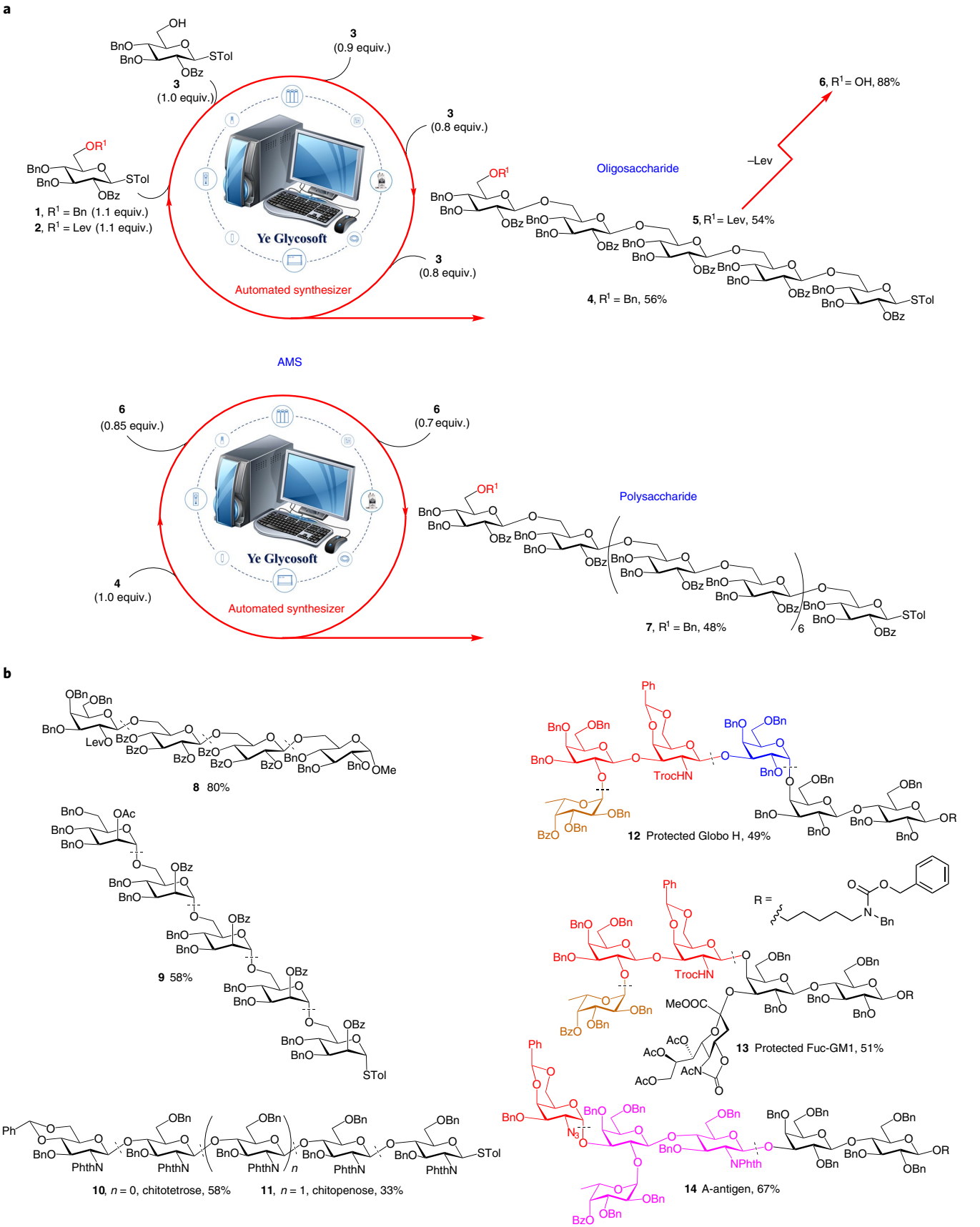

Fig. 3 | Automated synthesis of bioactive oligosaccharides in general activation mode. a, AMS was used to obtain oligosaccharides, such as **5** (1 × 5 = 5 mer) and polysaccharide **7** (1 × 5 × 3 = 15 mer). **b**, Automated synthesis of more oligosaccharides with various building blocks represented by different colours. The general activation mode used *p*-TolSCl/AgOTf or Ph_2SO/Tf_2O as activator. Ac, acetyl; Bn, benzyl; Bz, benzoyl; Troc, 2,2,2-trichloroethoxycarbonyl; Phth, phthaloyl.

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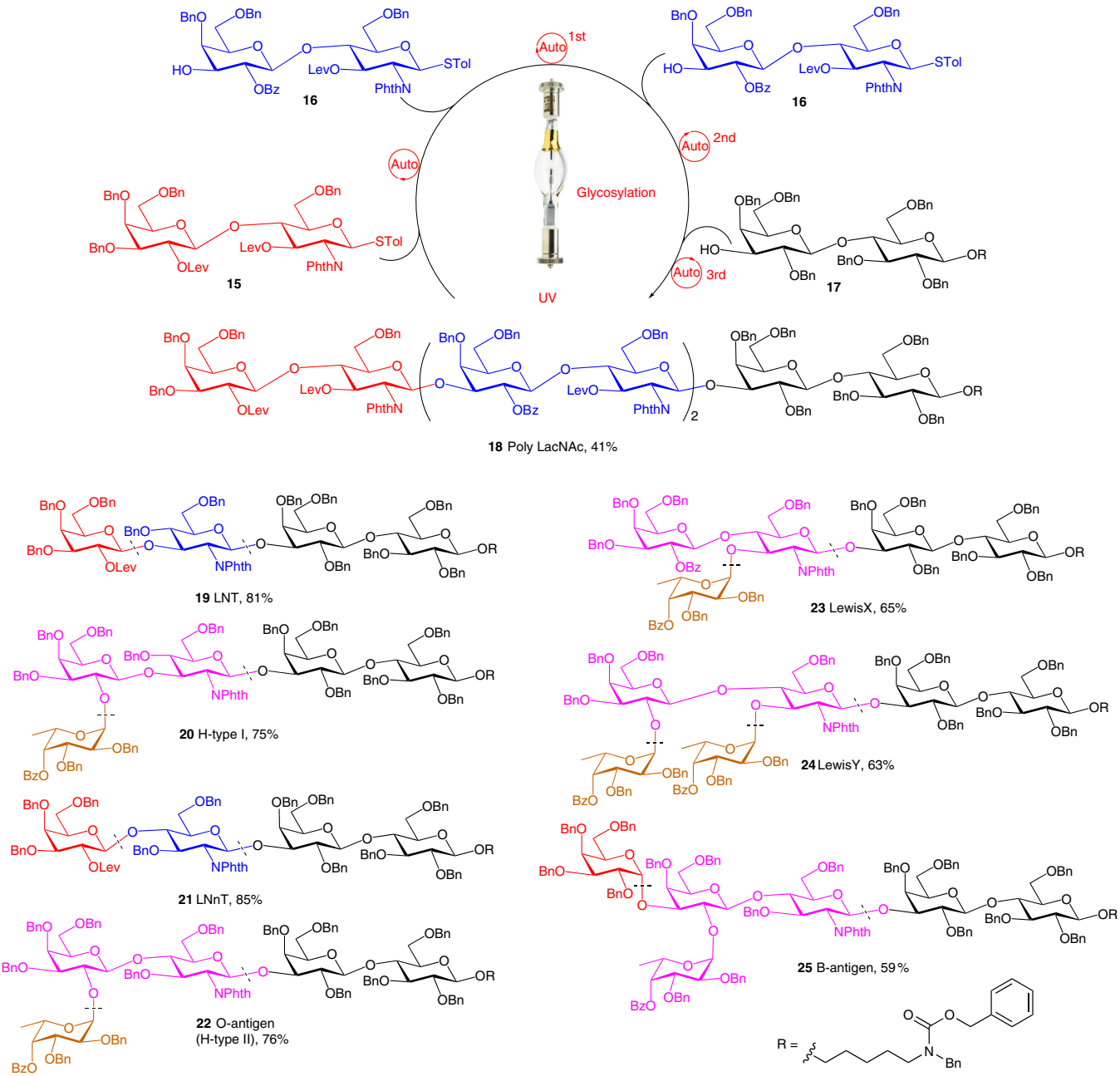

Fig. 4 | Automated synthesis of bioactive oligosaccharides in light-induced activation mode. Various building blocks are represented by different colours to show different components. The light-induced activation mode used Umemoto's reagent as photoactivator, $Cu(OTf)_2$ as additive and was carried out under ultraviolet irradiation. LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose.

lacto-*N*-neotetraose, are the core structures of human milk oligosaccharides and glycosphingolipids[32], and can be α-fucosylated at various positions to generate an array of biologically significant antigens such as H-type I, Lewis^X, Lewis^Y and ABO blood group antigens. In consideration of the biological importance and structural complexity of these antigens, the protected lacto-*N*-tetraose, lacto-*N*-neotetraose, H-type I, Lewis^X, Lewis^Y, B- and O-antigens **19**–**25** were accomplished using a similar three-component glycosylation strategy in automated photomediated synthesis mode. The automated synthesis of these useful antigens proves the efficacy of the synthesizer in its light-induced mode.

Automated synthesis of protected fondaparinux pentasaccharide

Fondaparinux (Arixtra), a unique antithrombin-III-binding pentasaccharide motif, was introduced to the market as an anticoagulant for the treatment of deep-vein thrombosis and acute pulmonary embolism due to its higher anti-Xa activity, longer half-life, better biosafety and a lower risk of heparin-induced thrombocytopenia compared with low-molecular-weight heparin[33]. However, the synthesis of fondaparinux (>50 steps) is very challenging despite the development of one-pot chemical synthesis[34,35] and chemoenzymatic synthesis[36], leading to high treatment costs and limiting its application. Here, the protected pentasaccharide **29**, an important precursor of fondaparinux, was synthesized efficiently by a [1 + 2 + 2] strategy using *p*-TolSCl/AgOTf as promoter and with the synthesizer equipped with a 25 ml reactor (Fig. 5). The synthesis used thioglycoside **26**, D-glucuronic-acid-containing disaccharide **27** with 3,6-di-*O*-acetyl groups for the α-directing glycosylation, and L-iduronic-acid-containing disaccharide **28** as building blocks, all of which were readily obtained from commercially available monosaccharide or disaccharide intermediates (see Supplementary

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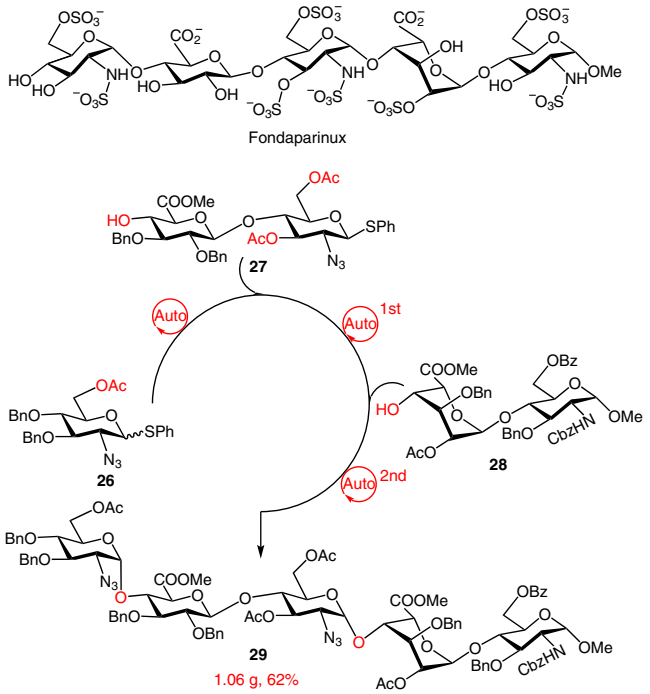

Fig. 5 | Gram-scale synthesis of a protected fondaparinux pentasaccharide. Reagents and conditions: (1) *p*-TolSCl, AgOTf, DCM/toluene, 4 Å molecular sieve, −72 °C to 0 °C, 3 h; (2) *p*-TolSCl, AgOTf, DCM/toluene, 4 Å molecular sieve, −60 °C to 0 °C, 3 h. Cbz, benzyloxycarbonyl; Tf, trifluoromethanesulfonyl; DCM, dichloromethane.

Information, section 2.4 for more details). Subsequently, by changing to a 100 ml reactor, the synthesis of pentasaccharide **29** could be conveniently scaled up (1.06 g) in 62% isolated yield, which is higher than that obtained by manual one-pot synthesis[34,35]. Thus, the automated synthesis of protected fondaparinux pentasaccharide has been achieved by an easily scalable reaction.

Automated multiplicative synthesis of arabinans up to a 1,080-mer

After synthesizing various oligosaccharides via three-component to five-component coupling reactions and the fondaparinux pentasaccharide from the milligram to gram scale, we shifted our attention to increasing the number of coupling components and the synthesis of polysaccharides of large molecular size[10,37]. One-pot glycosylation reactions involving up to seven-component coupling reactions have been reported[38]. Arabinans, which are composed of D-arabinofuranosyl (Ara*f*) residues and are essential structural constituents of plant and pathogenic bacterium cell walls[25], were chosen as the synthetic targets.

Using our synthesizer, with *p*-TolSCl/AgOTf as the promoter, the arabinan hexasaccharide **32** was assembled smoothly by a six-component, one-pot glycosylation (1 × 6 = 6) of monosaccharides **30** and **31** in 72% isolated yield on the gram scale. Subsequently, by further increasing the component number of the streamlined coupling reaction, heptasaccharide **33** (1 × 7 = 7), octasaccharide **34** (1 × 8 = 8) and nonasaccharide **35** (1 × 9 = 9) were obtained efficiently via multicomponent reactions in 63%, 58% and 53% isolated yields, respectively (Fig. 6a). Encouragingly, the decasaccharide **36** was synthesized automatically by a ten-component coupling reaction (1 × 10 = 10) using monosaccharides **30** and **31** as building blocks via nine streamlined glycosylation reactions, in an isolated yield of 47%, meaning an average yield of 92% for each single glycosylation. The reaction process was monitored by the online HPLC, which clearly displayed the coupling state of the reaction mixture after each glycosylation cycle (Fig. 6b). This one-pot, ten-component reaction is difficult to achieve by manual synthesis but can be realized with our synthesizer.

Based on the successful synthesis of hexasaccharide **32** (Araf_6) with an automated coupling procedure (1 × 6 = 6) starting from monosaccharides **30** and **31**, the assembly of longer arabinans by AMS was attempted. The AMS protocol is a multiplicative growth approach towards the synthesis of the desired polysaccharide via repetition of a cycle of two transformations (Fig. 7). These transformations are: first, the automated, multicomponent, one-pot coupling reaction of the glycosyl donor and acceptor (using *p*-TolSCl/AgOTf as promoter) to obtain the new intermediate donor (glycosylation); and second, selective removal of the temporary protecting group (*tert*-butyldimethylsilyl (TBS) group) at the non-reducing end of the intermediate donor to afford the new glycosyl acceptor (desilylation), which was performed manually. In this way, arabinans including 30-mer (Araf_{30} donor **38** and acceptor **39**, 1 × 6 × 5 = 30) and 120-mer (Araf_{120} donor **40** and acceptor **41**, 1 × 6 × 5 × 4 = 120) were rapidly constructed on the gram scale. Gratifyingly, the 360-mer arabinan **42** (Araf_{360}) was furnished efficiently by an automated three-component [**40** + **41** + **41**], one-pot coupling reaction (1 × 6 × 5 × 4 × 3 = 360). Subsequently, the coupling reaction of the Araf_{360} donor **42** with a large excess of 1-octanol afforded **44** in 85% yield. The removal of the TBS group in **42** and **44** with tetrabutylammonium fluoride (TBAF) provided the corresponding 360-mer arabinans **43** and **45** smoothly. With these 360-mer building blocks in hand, a subsequent automated three-component [**42** + **43** + **45**], one-pot glycosylation was performed. In spite of suffering from low reactivity and steric hindrance from the bulky size of both donor and acceptor, after screening and optimizing the reaction conditions, the [360 + 360 + 360] coupling reaction was, surprisingly, successfully realized in 33% isolated yield. Thus, the complete automated synthesis of the linear 1,080-mer polyarabinoside (Ara$f_{1,080}$-mer **46**, molecular weight 352,700) was accomplished via a five-step multiplying amplification approach (1 × 6 × 5 × 4 × 3 × 3 = 1,080). Finally, the fully protected arabinans can be completely deprotected, as exemplified by the global deprotection of polysaccharides **45** and **46**. After removal of the TBS group, the deacylation of polysaccharides **45** or **46** was completed under alkaline conditions. Subsequent purification by size-exclusion chromatography gave a residue, which was monitored by ^{1}H NMR spectroscopy to observe whether the signal of the benzoyl group appears (the chemical shift is around 8.0 ppm). For polysaccharide **45**, the next hydrogenolysis was executed once; for polysaccharide **46**, the hydrogenolysis was executed twice, because ^{1}H NMR spectroscopy showed that there was still a signal from the benzyl group after the first hydrogenolysis. It should be noted that the water solubility of polysaccharide **48** is poor, and it needs to be dissolved in hot water and purified at 35 °C. Thus, the global deprotection of polysaccharides **45** and **46** afforded the 360-mer arabinan Araf_{360} **47** and the 1,080-mer arabinan Ara$f_{1,080}$ **48** in 49% and 36% yields, respectively.

Conclusions

A dual-mode, automated, solution-phase glycan synthesizer using preactivation-based, one-pot, multicomponent glycosylation for carbohydrate synthesis has been made. Using this synthesizer, a variety of biologically useful glycans from oligosaccharides to polysaccharides have been assembled smoothly, either in general promoter-activation mode or in light-induced activation mode. The synthesizer can also efficiently synthesize a fondaparinux pentasaccharide on the gram scale. Furthermore, a one-pot, ten-component coupling reaction has been realized with the synthesizer, and, using AMS, an astonishing 1,080-mer arabinan with a well-defined structure has been constructed successfully; this arabinan is among the largest and longest polysaccharides/biomacromolecules ever synthesized. Our work provides a powerful approach to reliably access desired homogeneous complex glycans, and is expected to have a major impact on glycobiology, carbohydrate-based therapeutics and materials science. In addition, this method

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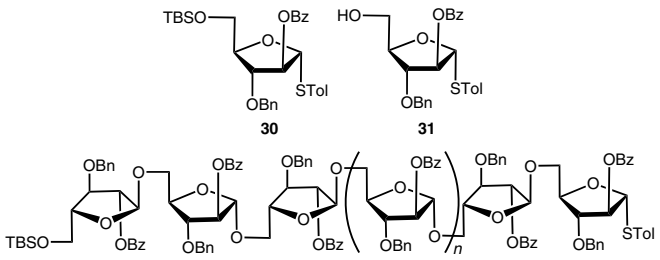

n	Components	Glycan	Yield (%)
1	6	Hexasaccharide **32**	72
2	7	Heptasaccharide **33**	63
3	8	Octasaccharide **34**	58
4	9	Nonasaccharide **35**	53
5	10	Decasaccharide **36**	47

b

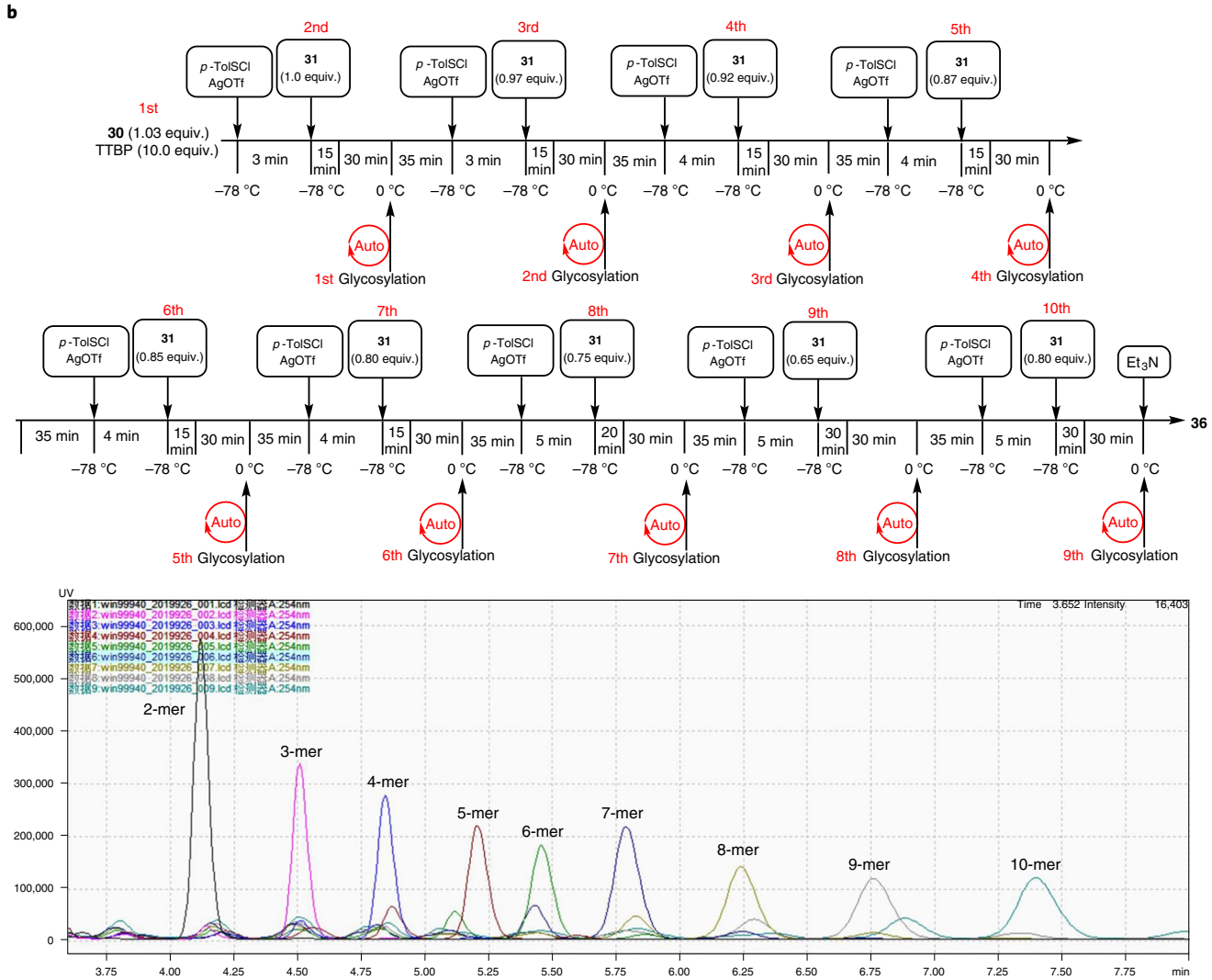

Fig. 6 | Automated multicomponent synthesis of arabinans. a, Six- to ten-component, automated, one-pot synthesis of arabinans. **b**, The ten-component automated, one-pot synthesis and online HPLC monitoring of decasaccharide **36** showing the conditions used over time. The HPLC traces show the results of the glycosylations and the increase in size of the arabinan after each glycosylation over time. The nine different colored lines represent the results of the nine glycosylations.

should be at least complementary to current automated solid-phase chemical and enzymatic glycan syntheses. This automated platform will not only benefit non-specialists, who will be able to assemble target glycans, but can also be applied to the streamlined synthesis of other organic molecules of interest.

Methods

The entire automated synthesis process comprises three parts: pre-automation, automation and post-automation. (1) Pre-automation: prepare various stock solutions and write the automatic synthesis program for the target molecule. Specific parameters and instructions can be modified, deleted, added, ordered and inserted as required; most of the instructions are modular or have been inserted according to our needs. (2) Automation: the synthesizer automatically executes the written project program in sequence (see Supplementary Information, section 2.2.1 for more details). The operator can pause or stop the automatic process if necessary. (3) Post-automation: preserve, summarize and analyse the automated synthesis results. The reactor is then disassembled and the reaction mixture subjected to manual separation and purification. The main differences between conditions used

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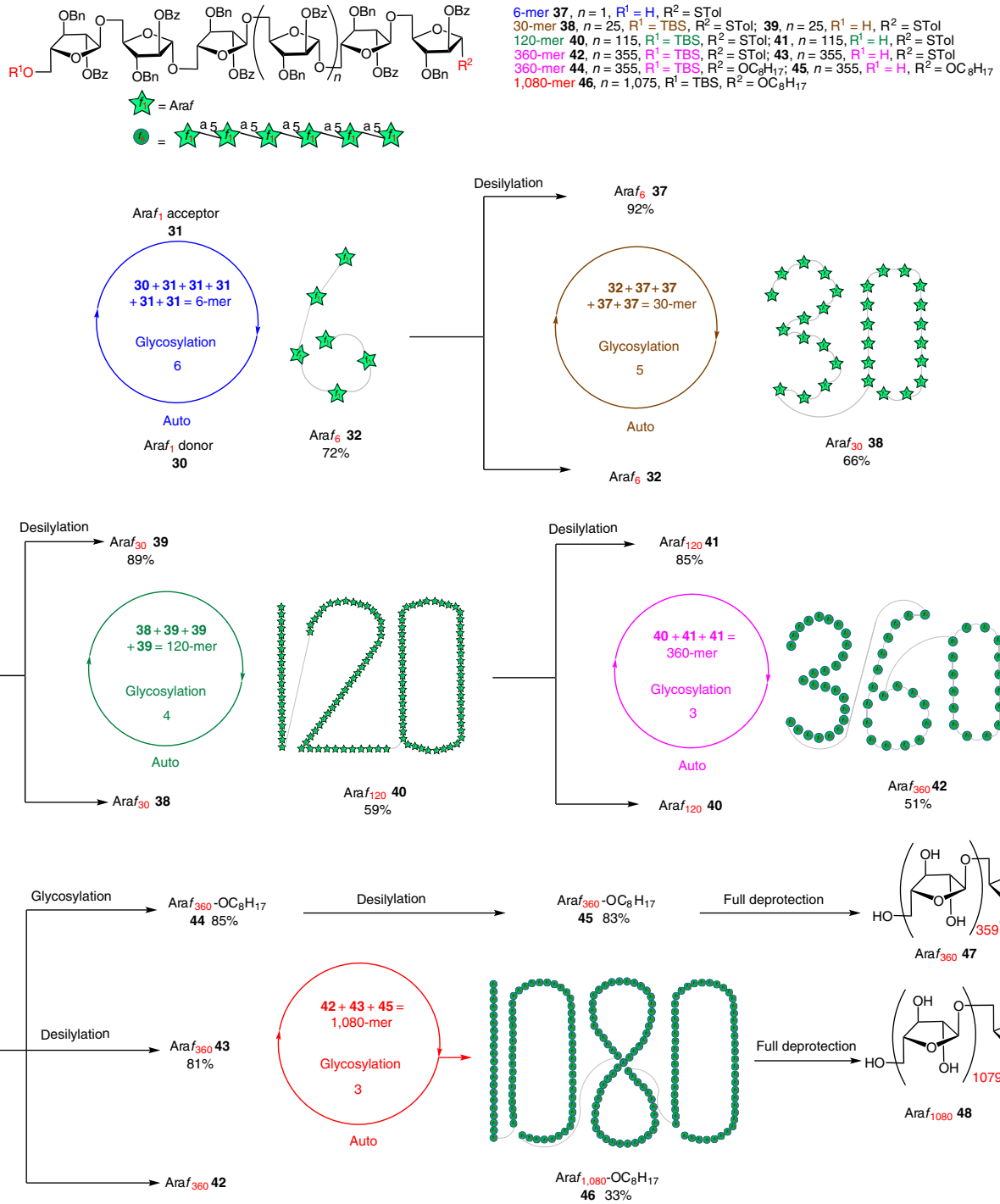

Fig. 7 | Automated multiplicative synthesis of linear 1,080-mer polyarabinosides (1 × 6 × 5 × 4 × 3 × 3 = 1,080) from monosaccharide building blocks. The images with green stars and circles are visual representations of the length of the polyarabinosides, where stars represent a single monosaccharide and circles represent six monosaccharides linked together.

for synthesizing different target compounds are activation system, activation temperature, reaction temperature and time, but the overall automated process is essentially the same. In the general activation mode, the activation system used is Ph_2SO/Tf_2O or *p*-TolSCl/AgOTf. In the light-induced mode, the activation system used is Umemoto's reagent, $Cu(OTf)_2$ and ultraviolet irradiation.

Data availability

The data reported in this paper are available in the main text or the Supplementary Information.

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Author contributions

X.-S.Y. conceived the research. W.Y., D.-C.X. and X.-S.Y. designed the experiments. W.Y. assembled the synthesizer and performed most of the synthetic experiments. Y.Y., C.G. and Z.C. assembled the synthesizer. F.L., B.-H.L., X.Q., L.-N.W., W.-Y.X., N.Y., H.Z., X.W. and M.L. synthesized monosaccharide and disaccharide building blocks. W.Y., D.-C.X. and X.-S.Y. analysed the data. W.Y. and X.-S.Y. wrote the manuscript. X.-S.Y. supervised the project.

Competing interests

X.-S.Y., W.Y. and D.-C.X. are applying for Chinese patents filed by Peking University. The other authors declare no competing interests.

Additional information

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