Accelerated Solid Phase Glycan Synthesis ASGS


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ABSTRACT: Solid phase synthesis is the most dominant approach for the synthesis of biological oligomers as it enables the introduction of monomers in an iterative, reproducible manner. Solid phase synthesis of oligosaccharides is crucial for the development of glycobiology. Accelerated solid phase synthesis of biological oligomers is crucial for chemical biology, but its application to the synthesis of oligosaccharide is not trivial. Solid phase oligosaccharide synthesis is performed in a variety of conditions and temperatures, requires inert gas atmosphere, and demand high excess of glycosyl donors. The reactions are usually very long, and the poor mixing of the solid support increases the risk of diffusion-independent hydrolysis of the activated donor. The entire process is slow and done mostly in special synthesizers. High shear stirring is a new concept in solid phase that enables shortening the synthesis time. The efficient mixing makes sure that reactive intermediates can diffuse faster to and into the solid support thereby increase the kinetics of the reactions. We report the first stirring-based accelerated solid-phase oligosaccharide synthesis. We harnessed high shear mixing to promote diffusion-dependent glycosylation over diffusion-independent side reactions resulting in high conversion in short reaction time. We eliminated the need for high excess of glycosyl donors and overcame the need to use inert atmosphere. We showed that by tailoring the deprotection and glycosylation conditions to the same temperature, all steps are performed continuously and full glycosylation cycles is completed in few minutes.

Introduction

Synthetic oligosaccharides are essential tools for glycobiology, vaccine development, therapeutics, cosmetics and more. The most essential step in oligosaccharides synthesis is the glycosylation of glycosyl acceptor by protected glycosyl donors to form a new glycosidic linkage. Glycosylation in solution is usually performed under very dry conditions and specific temperatures to ensure that the new glycosidic linkage can be formed without losing much of the reactive donor to undesired side reactions like hydrolysis. In most of those reaction, fast magnetic stirring is used to increase the diffusion of all the components in an attempt to complete the process with equimolar level of donor and acceptor (Fig 1 i). Solution phase oligosaccharide synthesis has to be stopped after each step to isolate the intermediate product before the next step which makes the process very lengthy and tedious. There are several routes for sequential synthesize oligosaccharide like the one-pot methods,[2] the fluoro-tag methods,[3] and several chemoenzymatic approaches [4]. However, the more common ways to prepare oligosaccharides in a continuous manner rely on solid support derived processes which are performed either in batch reactor or using flow through systems.[5] In those methods, the solid support is either stationary (flow-through) or mixed very poorly via argon bubbling.[6, 7] The efficiency of solid phase peptide and glycans processes is determined by a competition between the interaction of the activated species (high-energy, unstable electrophile) with the anchored moiety (usually nucleophile) and the decomposition of those unstable species. The poorly mixed solid phase reactions require the use of high excess of the reactive species and extended reaction time to ensure that all the anchored accepting moieties on the support have reacted.[5, 6] Such compromises increase the overall time of the process and makes the entire synthesis highly wasteful, especially for expensive glycosyl donors (Fig 1 ii). We discovered that much of those shortcoming are related to the fact that only poor mixing methods are used for SPS manipulation.[6] In those diffusion-deficient processes, increasing time and donor concentrations compensates for the decomposition of the active species that is usually diffusion-independent. The development of accelerated and efficient solid phase synthesis of biological polymers is essential for producing new biochemical tools. Since oligosaccharide solid phase synthesis takes place in reactors with poor mixing, by ramping between low and high temperatures, under inert atmosphere and using high excess of expensive glycosyl donors, accelerating the process is essential. Our previous studies showed that high shear mixing of the solid support enables the accelerated and efficient synthesis of peptide without breaking the solid support.[8]

We developed here the Accelerated Solid phase Glycans Synthesis (ASGS), a stirring-based SPS process for a record-fast preparation of oligosaccharides. The process relies on high shear mixing which allowed us to perform all steps at a constant
temperature and in semi-dry conditions thus enables completing an entire glycosylation/deprotection cycle in 8 minutes (Fig 1 iii). The new process was demonstrated by several mono and disaccharide syntheses in much shorter reaction time and fewer washing steps.

i. Stepwise solution phase glycosylation

Hours to days per cycle

Fast diffusion
Low donor excess
Non continuous
Manual
Many purifications

ii. Solid phase glycan synthesis: poor mixing + cooling/heating cycles

1 hour per cycle

Continuous
One purification
Can be automated
High donor excess
Slow diffusion
Long process

iii. Accelerated Solid phase glycan synthesis: fast mixing + constant temperature

8 min per cycle

Continuous
One purification
Low donor excess
Fast diffusion
Short process
Can be automated

Results and discussion

In this work, we aim to establish a new strategy for synthesizing oligosaccharides using a new reactor setup with fast overhead stirring (Fig 1iii). The cooling of the reaction was achieved via a double jacket reactor and the temperature was maintained by external cooling bath. The solvents of the glycan donors, the activator solution and the washings were all dry but the reaction was performed in ambient atmosphere without inert gas flow.

The idea was to rely on the fast kinetics, enabled by the reactivity of the glycosyl donor and the fast stirring, to achieve high conversions even in ambient atmosphere. We assume that the fast-stirring assisted glycosylation of the solid support can overcome diffusion-independent hydrolysis of these donor even in an open vessel. Two linkers and five different donors were used in the study to enable screening of different protecting group’s hierarchy and the leaving groups (Scheme 2A).
Accelerated glycosylation of high loading resin under ASGS conditions

The glycosylation was done in a reactor setup (Fig S1) with fast overhead stirring cooled to 0 °C under ambient conditions without using inert gas. The high loading photolabile linker support 1 was glycosylated with four different thioglycoside donors (3-6) to yield glycosylated linker 3a-6a (Fig. 2B). In the fast stirring glycosylation protocol, we injected 1 equiv of the donor to the reactor which was followed by the immediate injection of activator. The reaction took place for 1 min at constant 1200 rpm stirring rate. As in previous works, the polystyrene beads were intact throughout process. The reaction was repeated two more times to complete the glycosylation process in 3 min using only 3 equiv of the donor. Two 10 second-washing-repeats were enough to remove remaining donor and activator from the resin. The glycosylated linkers 3a-6a were cleaved off using the breaking-beads photocleavage approach,[7] and the crude mixtures were analyzed without further purification by HPLC to determine the conversion ratio and by 1H NMR, 13C NMR and HSQC to validate products integrity. In all cases, the glycosylation under ASGS conditions provided almost complete conversion of the linker to the glycosylated linker. This proved that even under ambient conditions and with very short time span and donor excess, an efficient glycosylation could be made. We assume that using three cycles with low equivalents allowed us to minimize the amount of hydrolyzed donor thereby increase the efficiency of the process. The small library also show that the conditions are suitable for donors that are derived from four monosaccharides types that are equipped with different protecting groups.

Glycosylation on low loading resin

To further challenge the system, we used lower loading resin 2 with the same donors (3-6) to synthesize glycosylated linkers 3b-6b (Fig. 2C). We used the same amount of resin so that the active concentration of reagents, donors and acceptor sites in the system was actually half the one used for the glycosylation of 1 (0.3 mmol/gr compared to 0.6 mmol/gr). HPLC analyses after cleavage showed over 95% conversion for all the donors proving that the stirring was efficient enough to enable fast glycosylation even at lower concentrations.

Glycosylation with different LG

To check if the system can be applied to other leaving groups, we converted glucosamine thioglycoside 3 to the corresponding N-phenyltrifluoroacetimidate 3' (SI). 3' was used to glycosylate solid support linker 2 using catalytic amount of TMSOTf as activator to provide 3b. For imidate glycosylation we applied the same short 1 min glycosylation repeats as used for the ASGS thioglycoside donors protocol (Fig. 2C and SI). After cleavage, the crude mixture was analyzed without further purification by HPLC to show 98% conversion. This demonstrates that ASGS can be used with various activation systems.

Thioglycosylation without stirring

The effect of stirring proved detrimental for Fmoc removal and amide bond formation in our previous work.[6] Here we wanted to evaluate the effect of stirring on glycosylation efficiency. The synthesis of 4b was attempted by reacting donor 4 with linker 2 under ASGS conditions, only that this time no stirring was applied (Fig. 2C). HPLC analysis after cleavage showed only 35 % conversion compared to almost complete conversion that was observed for the same ASGS glycosylations with stirring. The deficiency of the reaction without stirring can be attributed to several facts. The formation of the active glycosyl intermediate was hampered because of slow diffusion of the activator and the donor. The diffusion of the activated glycosyl donor to and into the bead-anchored acceptor was too slow for the glycosylation to reach completion in such short time span. The diffusion-independent hydrolysis of the activated donor in the open vessel can be significant by product without stirring hence decrease the concentration of activated donor.

ASGS Fmoc deprotection optimization

The temperature, the base concentration, the base type, and the mixing efficiency have significant effect on Fmoc removal rate. Decreasing the temperature of the reactor to 0 °C can pose a real limitation for the short reaction periods used in ASGS since the temperature affects both the kinetics of the reaction and the diffusion of reagents. To test the efficiency of the Fmoc deprotection, we attempted the removal of Fmoc from O-4 hydroxyl group of glucosamine 3a anchored to high loading linker 1 at 0 °C using the standards 20% piperidine/DMF solution. Analysis of the resulting crude proved that only 9 % of the Fmoc was present.
removed to provide 3c at 0 °C using 20% piperidine even when fast stirring was applied (Fig. 3). This showed that the standard protocol cannot work in ASGS fast cycles that takes place at low temperatures. To improve the Fmoc deprotection step at 0 °C we used a combination of 20 % piperidine and 5% 1, 8-Di-azabicyclo 5.4.0 undec-7-ene (DBU) in DMF. DBU is a strong base that is often used to increase the efficiency of Fmoc deprotection during peptide synthesis.\cite{8} Using the piperidine/DBU combination, Fmoc deprotection from 3a provided exclusively 3c under low temperature, fast stirring and short reaction times as in ASGS conditions (Fig 2). To confirm the generality of the protocol, Fmoc was removed from O-6 of galactose 4b anchored on low loading solid support 2 to provide the deprotected 4c by using pip/DBU in DMF. HPLC show full Fmoc deprotection also in the low loading linker and from primary hydroxyl position of 4b (SI).

Fig 3. Fmoc removal in ASGS by 20% piperidine/DMF solution (up) and 20 % piperidine + 5 % DBU/DMF solution (down).

**ASGS disaccharides synthesis**

After optimizing the condition of Fmoc deprotection we synthesized 1-6-β-di-galactose disaccharide 4d on linker 2 (Fig. 4) via a combination between thioglycoside glycosylation protocol and the pip/DBU Fmoc deprotection protocol (SI). One equiv of 4 was inserted to the reactor (pre-cooled to 0 °C) which was followed by the injection of same amount of activator. Glycosylation was completed in a minute and was repeated two more times to ensure full conversion to 4b. After fast-washing steps, Fmoc was removed at the same temperature using two short 2 min reactions to give 4c. A single DMF wash was enough to complete the entire cycle process in less than 8 min. Glycosylating 4c with donor 4 under the same conditions as in the first cycle provided crude 4d. The entire process for the synthesis of 4d was about 10 min and the analysis proved that the crude product was obtained in over 90% purity (SI).

To further prove the generality of the process, 1-6-β-di-mannose 5d was synthesized on the higher loading linker 1 (loading 0.6) using donor 5 and the corresponding glycosylation and deprotection protocols (Fig 3 and SI). Here again the synthesis of the 5b was completed in about 11 min in and yielded the di-mannose disaccharide in over 90 % crude purity. The synthesis of 4d and 5d on two different linkers using similar reaction cycles and identical time and temperature suggests that the process can be reproduced for other glycans. Cooling slows down reactions and have a significant effect on diffusion dependent processes. The activated glycosyl donor species are short-lived. The risk of excessive donor hydrolysis due to moisture accumulation in a reactor that operates at low temperatures in non-inert conditions is realistic and dictates a different strategy to enable glycosylation. We applied short glycosylation repeats with low molarity. Enhancing the diffusion by high shear mixing can increase the chances that the activated donors will reach the acceptor on the solid support before they decompose/hydrolyze.

This is a new concept to overcome the use of excessive donors and can enable very fast reactions that favors glycosylation (diffusion-dependent) over hydrolysis (diffusion-independent). The use of a single temperature operation also calls for a change in standard reaction protocols. The most relevant example showed here was the inadequacy of the common 20% piperidine/DMF solution to remove Fmoc from glycans in short time under low temperature. While such conditions are mostly used to remove Fmoc at longer reaction times and higher temperature, they failed to fit the ASGS process. The DBU piperidine combination provided the suitable solution to enable the removal of Fmoc at the low temperature and fast reactions required for ASGS. The use of a single temperature for all manipulations allowed us to perform all the steps without stopping the process for cooling or heating thereby accelerating the overall synthetic time. In this work, we used donors that provides trans glycosylations to synthesize short linear glycans in order to demonstrate the feasibility of the approach. Further development of the setup and the optimization of the synthetic conditions will allow in the future to synthesize large, branched, cis linked glycans in short time span.

Fig 4. ASGS of disaccharides 4d and 5d.
ASSOCIATED CONTENT
Supporting Information
Experimental Procedures, Synthesizers modules, HPLC, NMR, MS, all are described in details in the SI.

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