Building up cyclodextrins from scratch - templated enzymatic synthesis of cyclodextrins directly from maltose

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Building up cycloextrinsics from scratch – templated enzymatic synthesis of cycloextrinsics directly from maltose

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Cycloextrinsics (CDs) are commercially produced via enzymatic breakdown of starch or amylose. In contrast, we show that cycloextrinsics can be synthesised directly from the disaccharide maltose in good yields by exploiting the use of templates to favour the enzymatic build-up of cycloextrinsics. Using cycloextrin glucanotransferase to catalyse reversible transglycosylation, the maltose in good yields by exploiting the use of templates to favour cyclodextrins can be synthesised directly from maltose in approximately 70% yield. This work represents a step towards supramolecular control over enzymatic production of complex oligosaccharides from simple building blocks.

The efficient production of oligosaccharides continues to be a challenge for the synthetic chemistry community. Stepwise syntheses, involving sequential protection, glycosylation and deprotection, can be impressive, but require highly-skilled synthetic chemists and are rarely scalable, and thus only a few therapeutically important oligosaccharides are produced synthetically on an industrial scale. In contrast, other biopolymers, oligonucleotides and proteins, are readily assembled using a combination of automation (e.g. DNA synthesis) and biotechnologies that employ template-controlled enzymatic synthesis (e.g. PCR and protein expression). Chemoenzymatic and automated approaches to oligosaccharide synthesis that harness the unique capacity of enzymes to control chemoselectivity and stereoselectivity have shown recent success. We are seeking to develop an approach that exploits enzymes to generate dynamic mixtures of oligosaccharides that interconvert via the reversible formation of glycosidic linkages, and employs synthetic templates to bind, select and amplify specific oligosaccharide products.

Cycloextrinsics (CDs) are macrocyclic oligosaccharides that exhibit truncated cone-like structures made up of typically 6, 7 or 8 (α, β, or γ-CD) d-glucopyranose units linked by α-1,4-glycosidic bonds. Due to the relatively hydrophobic nature of the CD cavities, and their concomitant ability to bind and the CD cavities, and their concomitant ability to bind and increase the aqueous solubility of poorly soluble compounds, they have found wide applicability within many fields, such as in the cosmetics, pharmaceutics, and food industries. Commercially available CDs are produced by treating biopolymers made up of d-glucopyranose units linked by α-1,4-glycosidic bonds (α,1,4-glucans), such as starch or amylose, with cycloextrin glucanotransferase (CGTase), which breaks down the biopolymer into primarily CDs and small amounts of linear oligosaccharides. The build-up of cycloextrinsics from monosaccharides using synthetic organic chemistry has been described but these are complex multistep-syntheses that are impressive, but inevitably low yielding. We have recently shown how the process by which CGTase makes CDs from an α,1,4-glucan source is dynamic and responsive. The enzyme scrambles the glycosidic linkages in linear and cyclic α,1,4-glucans enabling the interconversion of CDs via a series of transglycosylation steps. The CDs in this dynamic system form as a kinetically-trapped subsystem that exists transiently before the eventual conversion of all glucan material to glucose (G1), the thermodynamic product. The CDs exist in what is essentially a transient Dynamic Combinatorial Library (DCL), and can interconvert via enzyme-mediated dynamic covalent chemistry. Using template molecules, we can control the selective interconversion of CDs. For example, we could successfully turn α-CD into β-CD by treating α-CD with CGTase in the presence of 1-adamantane carboxylic acid, a compound that binds strongly and selectively to β-CD.

In the present communication we show that by exploiting template effects, CDs can be synthesised directly from maltose (G2) (Fig. 1). Our approach of building up CDs from small building blocks is the reverse process of the polymer breakdown used in current CD production, and represents a step towards supramolecular control over enzymatic production of complex oligosaccharides from simple carbohydrate building blocks.
Maltose (G2), and not glucose (G1), is the simplest building block from which CDs can be assembled by CGTase. CGTase scrambles α,1,4-glycosidic linkages in a two-step process. Firstly, a covalent enzyme-substrate glycosyl donor complex is formed by cleaving one α,1,4-glycosidic linkage in the substrate, and if the substrate is non-macroyclic, releasing α-glucose (G1) or a longer α,1,4-glucan. Secondly, a new α,1,4-glycosidic linkage is formed when the enzyme is displaced by a glycosyl acceptor. If this second step involves an intramolecular reaction, a CD is formed, and if it is intermolecular a new linear α,1,4-glucan is formed. In the case of G2 as a substrate, the reducing end (hemiacetal) α-glucopyranosyl unit (illustrated with black rectangles in Fig. 1a) can thus be considered a “leaving group.” To form 1 mole of α-CD, 6 moles of G2 would thus be required and 6 moles of G1 are generated as a by-product (see section S2, ESI).

To investigate whether or not the inexpensive, readily available, and highly soluble maltose (G2) could be utilised to synthesise CDs, we used NMR spectroscopy. CGTase (0.57 mg/mL) was added to a solution of G2 (10 mg/mL) in D2O buffered with sodium phosphate (50 mM, pH 7.5) at 25 °C. The relative total concentration of CDs was quantified by comparing the integrals for anomeric (H1) signals of the CDs (H1CDs, highlighted in blue in Fig. 2a, 5.06 – 4.96 ppm), the reducing-end (hemiacetal) α-anomer of the linear oligomers (α-H1red,endo, highlighted in yellow, 5.18 – 5.13 ppm) and the α-1→4 linked internal (acetal) units of the linear oligomers (H1internal, highlighted in red, 5.36 – 5.28 ppm) (Fig. 2a). Some CD formation was observed, but the conversion was poor, and a maximum molar yield of 10% was obtained after 1.5-2 hours. We report the molar CD yield taking into consideration that only acetal glucopyranosyl units can be incorporated into CDs. Therefore, molar yield = 100% × [6(α-CD) + 7(β-CD) + 8(γ-CD)]/((n-1)[G1]0) where [G1]0 is the concentration of the linear starting material used in the reaction and n is the number of α-glucopyranosyl units in the starting material (i.e. n = 2 for G2). The relative concentrations of α, β, and γ-CD were not determined at this point, as their signals are not sufficiently well resolved using 1H NMR, but from previous experiments we expected all three would form.α,β,γ

CD production from G2 did not start immediately (Fig. 2b, grey trace). We observed an initial lag-phase, which can be explained by the fact that a number of intermolecular transglycosylation steps are required in order to build up linear α-glucans that are long enough to produce CDs via an intramolecular transglycosylation. At least 6 transglycosylation steps are required to convert G2 to α-CD (see section S2, ESI). The build-up of longer α-glucans during the first 30 mins is seen in the broadening of the H1 signal from the internal α-1→4 linked α-glucopyranosyl units (5.36 – 5.28 ppm), due to the presence of several overlapping signals (Fig. 2a, left).

We initially thought that a more efficient conversion of G2 to CDs could perhaps be achieved by ‘spiking’ our reactions with small amounts of longer linear α-glucans or CDs in order to reduce the number of transglycosylation steps required to convert the G2 to CDs. G2 (9 mg/mL) was thus treated with CGTase in the presence of α-CD (1 mg/mL) (Fig. 2b and section S3, ESI). Although the lag-phase was indeed shortened by ‘spiking’ the reaction with α-CD, the large excess of short glucosyl acceptors rapidly converted the α-CD to short linear oligosaccharides resulting in a CD yield increase that was lower than the amount of CD spike (section S3, ESI).

Having previously shown that templates can promote the selective synthesis of CDs from maltohexaose (G6) by forming...
specific stabilising host-guest complexes, we hypothesised that higher conversion of G2 to CDs might be promoted by addition of a template. G2 (10 mg/ml) was therefore treated with CGTase (0.57 mg/ml) in the presence of sodium dodecyl sulfate (SDS) (5 mM), 1-adamantane carboxylic acid (ACA) (10 mM) or sodium tetraphenylborate (NaBPh4) (10 mM), known to bind α-CD, β-CD and γ-CD, respectively. In addition to reaction monitoring by 1H NMR spectroscopy, high performance liquid chromatography (HPLC) coupled to an evaporative light scattering (ELS) detector was utilised in order to ascertain the specific CD composition formed. Calibration curves from 0.01 mg/ml to 10 mg/ml for all three CDs as well as the linear α-1,4-glucans G1 to G8 were made in order to correct for differences in the response of ELS detector to different oligosaccharides (S1, ESI).

In the presence of templates, we saw remarkably high levels of CD synthesis from G2 (Fig. 2a,b and Figs. S12, S13, ESI). The yields and distribution of CD products are shown in Figure 3a. Most impressively, β-CD was synthesised from G2 in 58 ± 4 % yield in 3 hours with > 95% selectivity using ACA as a template. To optimise the selective synthesis of β-CD from G2, we examined the influence of template concentration and starting G2 concentration. By increasing the concentration of ACA template from 10 mM to 20 mM, the yield increased to 67 ± 2 % (Fig. 3b). By decreasing the starting concentration of G2 to 5 mg/ml we obtained a further improved yield of 70 ± 3 % in the presence of 20 mM ACA (Figs 1b, 3c and Fig. S14, ESI). We propose that the increased conversion of G2 to CDs in the presence of templates is due to a kinetically controlled “capture” mechanism. As soon as CDs are formed, they are bound by the templates, thus stabilising them and preventing their conversion back to short linear α-1,4-glucans, and ultimately glucose.

In a true dynamic combinatorial library operating under thermodynamic control, the product distribution at equilibrium should be dependent only on the available building blocks and not on the specific starting material. In contrast, since this dynamic system forms CDs out-of-equilibrium in a kinetic trap, the choice of starting material is significant. It is noteworthy that the highest yield of CDs from G2 in the absence of templates is 10% while we have previously observed yields of over 60% for the same reaction started from G6. To better understand this effect, each of the linear α-1,4-glucans ranging from maltose (G2) up to maltooctaose (G8) was treated with CGTase in the absence of template and the reactions monitored over time using HPLC-ELS (Fig. 4a,b and Figs. S15 - S17, ESI).

The molar CD yield was found to increase, and the time to reach maximum CD yield to decrease, as the length of the oligosaccharide starting material was increase (Fig. 4a). There appears to be an inverse correlation between the CD yield and the number of transglycosylation steps required to form CDs from the starting material, making G2 the most challenging substrate. In all cases it was observed that once formed, a steady concentration of medium length (G4-G6) and long (G7 and G8) α-1,4-glucans is observed, while CDs are first built up and then slowly converted into the short linear α-1,4-glucans (G1-G3) (Fig. 4b). For G2-G6, which must first undergo at least one intermolecular transglycosylation to form a longer linear α-1,4-glucan before cyclisation to form CDs is possible, we expect a lag-phase before CDs begin to form. This is not immediately evident from Fig. 4b for G4 and G6, but can in fact be observed for G6 when the reaction is analysed with very high time resolution (Fig. S18, ESI). In contrast, when the reaction is started from G7 and G8, CDs can form directly, resulting in higher quantities of α-glucan material being kinetically trapped as CDs and therefore the higher CD yields.

To summarise the workings of the system we propose the generalised energy diagram depicted in Fig. 4c. The short linear α-glucans (G1-G3) represent the thermodynamically more stable products (with G1 being the final product upon reaction ad infinitum), while the CDs exist in a kinetically trapped subsystem. Exchange of α-glucopyranose building blocks between these two extremes must go via the medium length (G4-G6) and long (G>6) linear α-glucans, which therefore represent higher energy intermediates. Long linear α-glucans are only ever observed in very low concentrations, as they can readily convert to CDs (Fig 4b). Upon addition of templates an additional, deeper kinetic trap is formed, which significantly boosts the CD yields, even when starting from G2. Meanwhile addition of a template to the reaction starting from G2 does not affect the lag-phase since longer α-1,4-glucans must first assemble before they can cyclise. The HPLC-ELSD data provide direct evidence of this, since the accumulation of higher quantities of kinetically-trapped CDs in the presence of template is first seen after the longer α-glucans (G7 and G8) are detected in the system, i.e. after approximately 30 minutes of reaction (Fig. 4d).

In conclusion, we have developed a method to enzymatically build up cyclodextrins from maltose, in contrast to the conventional method which relies upon the breakdown of polysaccharides. In the absence of templates, this route is inefficient and low yielding. In combination with templates, dramatically improved CD yields can be obtained (up to ca. 70% in the case of the ACA template). The overall CGTase- mediated
System of α-glucans is dynamic but CDs are formed out-of-equilibrium, which means that the choice of starting material has an influence on the CD yield. Exploiting knowledge of this dynamic system allows us to use templates to kinetically trap CD products that are otherwise formed transiently and sparingly, in order to favour their production profoundly. This system represents a first step towards the use of dynamic oligosaccharide systems to produce higher value and more complex oligosaccharides from simple building blocks by stabilising them with supramolecular interactions.

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Conflicts of interest
There are no conflicts to declare.

Notes and references
10. Where the CD yield was determined by both NMR spectroscopy and HPLC-ELS, it is reported as the average of the two determinations ± one standard deviation.