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Unnatural cyclodextrins can be accessed from enzyme-mediated dynamic combinatorial libraries

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Dynamic systems of cyclodextrins (CDs) enabled by a native cyclodextrin glucanotransferase (CGTase) can incorporate unnatural glucopyranose-derived building blocks, expanding the applicability of enzyme-mediated dynamic combinatorial chemistry by using synthetically modified substrates. Starting dynamic combinatorial libraries from CDs with a single 6-modified glucopyranose results in a dynamic mixture of CDs containing several modified glucopyranoses. The relative concentrations of modified α, β or γ-CDs can be controlled by the addition of templates, providing a novel way to access modified CDs.

Enzyme-mediated dynamic combinatorial chemistry uses enzymes as catalysts to make dynamic combinatorial libraries (DCLs) of interconverting oligomers.[1–10] Due to the natural origins of the enzymes, the building blocks for these oligomers are natural biomonomers, such as amino acids[1–4] or carbohydrates.[5–10] Such dynamic systems have enabled the development of peptide-based nanomaterials from protease-catalysed systems[2–3] and the exploration of light-controlled dynamic systems of cyclodextrins (CDs), where the relative ratios of α, β and γ-CD can be controlled by an external light source.[9-10]

Although enzymes are often considered highly specific catalysts, many enzymes also exhibit some form of catalytic or substrate promiscuity.[11] Recent decades have seen an increase in the exploitation of enzyme promiscuity, including the use of the inherent promiscuity of native enzymes, as well as the engineering of enzymes to be able to perform reactions otherwise not seen in Nature.[12–13] We reasoned this promiscuity could be exploited to make DCLs containing both natural and synthetically modified biomonomers.

Native CDs consist of 6, 7 or 8 D-glucopyranose units (α, β or γ-CD, respectively) joined by α-1,4-glycosidic linkages to form a macrocycle with a truncated cone-like structure and a hydrophobic internal cavity.[14] CDs form host-guest complexes with hydrophobic guests in water, which has been widely exploited in supramolecular chemistry and catalysis,[14-16] as well as in the pharmaceutical, food and cosmetics industries.[17] Modified CDs, including mixtures of CDs, can sometimes be more efficient than a single native CD for these encapsulation applications. For example, Febreze® is composed from a mixture of various cyclodextrins that is particularly efficient at

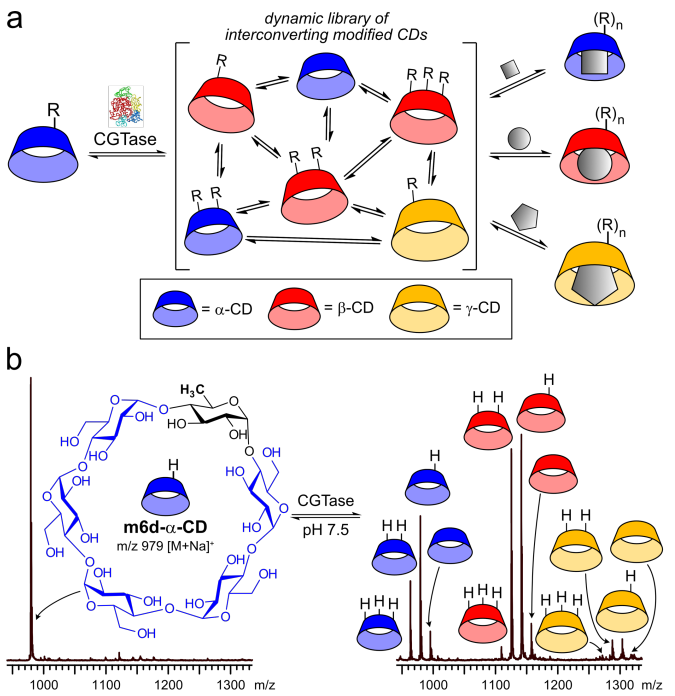

Fig. 1 Enzyme-mediated dynamic combinatorial libraries of modified CDs. (a) Dynamic libraries of CDs with modifications (R) on C-6 can be produced by treating a CD containing a single modified D-glucopyranose unit with CGTase. Addition of templates shifts the equilibrium to produce more of the desired size of modified CDs. Equilibrium arrows inside the square brackets illustrate that all library members can interconvert but are not meant to indicate specific direct transformations. (b) Treatment of **m6d-α-CD** with CGTase results in a DCL containing α, β, and γ-CDs with several 6-deoxyglucose units (MALDI-MS, pH 7.5, 10 % by vol. DMF, after 30 h at 22 °C).

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eliminating odours.[18] Mixtures of modified CDs can encapsulate and solubilise pharmaceutically active substances,[19] and have been employed to resolve enantiomers,[20] among other biological applications.[21] In aqueous biphasic organometallic catalysis, synergistic effects have been observed by using mixtures of CDs as mass transfer agents.[22] The production of modified cyclodextrins for specific applications remains a challenge although complex synthetic procedures have recently emerged.[23] Straightforward synthetic approaches could lead to breakthroughs in this field.

Cyclodextrin glucanotransferase (CGTase) is employed in the enzymatic synthesis of CDs from starch. CGTase catalyses the reversible inter- and intramolecular transglycosylation of α-1,4-glucans, to give linear and cyclic (cyclodextrin) products, as well as slow background hydrolysis.[5] Several reports have exposed the inherent promiscuity of this class of enzymes, but the investigation of CGTase-catalysed synthesis of modified CDs has received only a little attention.[24-25]

We have previously shown that CGTase-catalysed DCLs of the natural (unmodified) CDs can be generated by exposing an α-1,4-glucan starting material to CGTase at room temperature. A dynamic mixture of interconverting CDs forms in a kinetically trapped subsystem that operate under pseudo-thermodynamic control, such that the distribution of α-, β- and γ-CDs formed reflects their relative intrinsic stabilities and any external stabilising influences (e.g. interactions with added templates).[5–8] In this report, we exploit the promiscuity of a commercial CGTase to produce DCLs of modified CDs. We show that these systems of interconverting (modified) CDs also operate under pseudo-thermodynamic control, and we demonstrate that templates can be used to control which modified CDs are preferentially produced (Figure 1a).

We envisioned that CDs with minor modifications, both in terms of the size and chemical traits of the modification, would be most likely to be accepted as substrates by CGTase. Therefore, we set out to synthesize mono-6-deoxy-α-CD (**m6d-α-CD**, Figure 1b) and mono-6-deoxy-β-CD (**m6d-β-CD**). These two new modified CDs were obtained starting from mono-6-tosyl-cyclodextrins, which were subsequently reacted with sodium borohydride in anhydrous DMSO using an adapted procedure[26-27] to give the desired compounds (ESI Section S1).

m6d-α-CD was found to be quite soluble (at least up to 25 mg/mL) in purely aqueous sodium phosphate buffer (pH 7.5), but **m6d-β-CD** was only sparingly soluble (less than 1 mg/mL). Organic co-solvents have previously been shown to reduce, but not eliminate the activity of CGTase.[6] Reaction conditions involving a number of co-solvents were thus tested, and the use of 10% DMF by volume in 40 mM sodium phosphate buffer (pH 7.5) at room temperature gave the best results with regards to CD solubility and enzyme activity (ESI Section S2). When **m6d-α-CD** (5 mg/mL) was treated with CGTase under these conditions, a dynamic mixture of α-, β- and γ-CDs each containing up to three 6-deoxyglucopyranose units was formed (Figure 1b). The reaction was monitored by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-MS) wherein the different CDs were observed as the $[M+Na]^+$ ions. Since MALDI-MS does not distinguish positional

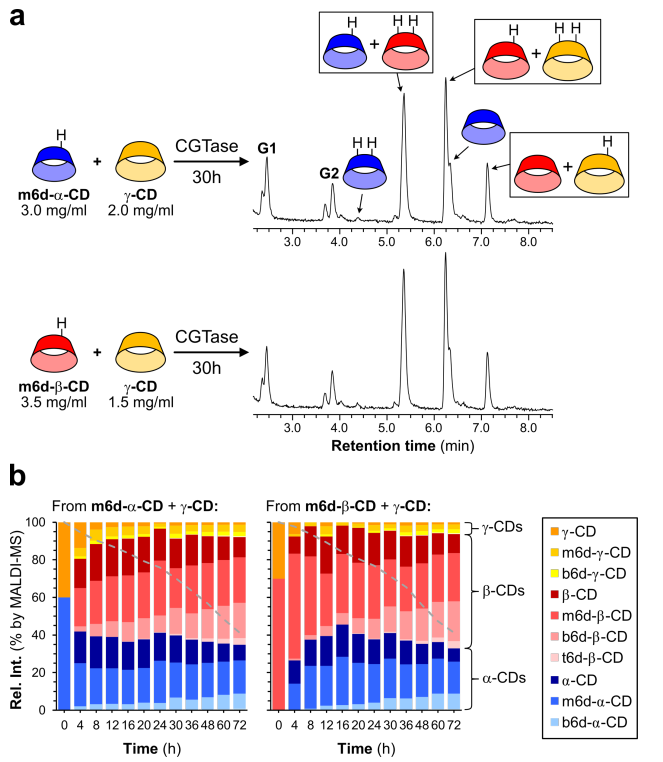

Fig. 2 Proof of pseudo-thermodynamic equilibrium. (a) The starting concentrations of two libraries started from different starting materials, but with the same overall building block concentration (5.0 mg/mL) and composition (9:1 glucopyranose to 6-deoxyglucopyranose), and chromatograms (HPLC-ELS) obtained after the libraries had converged (30 hours). (b) Relative intensities of all observed CDs (MALDI-MS) in the same two libraries. The total CD concentration (as evaluated by HPLC-ELS) is illustrated with a grey dashed line (% by weight out of all α-1,4-glucans). Reaction conditions as in Fig. 1b.

isomers, we will refer to CDs with one, two and three deoxyglucopyranose units non-specifically as mono, bis and tris-6-deoxy-CDs (m6d-CDs, b6d-CDs and t6d-CDs), respectively.

To demonstrate that this mixture of modified CDs was dynamic, and that the system of CDs was operating under pseudo-thermodynamic control, two DCLs containing identical combinations of building blocks were prepared from two different starting points and monitored to see if they would converge on the same composition (Figure 2). The first DCL was prepared from **m6d-α-CD** (3.0 mg/mL) and γ-CD (2.0 mg/mL), and the second was prepared from **m6d-β-CD** (3.5 mg/mL) and γ-CD (1.5 mg/mL). In both cases, the ratio of glucopyranose to 6-deoxyglucopyranose units present was 9:1, and, therefore, when exposed to CGTase, the DCLs should eventually reach identical compositions at pseudo-thermodynamic equilibrium. The evolution of each DCL was monitored using a combination of MALDI-MS and high-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELS). The identities of the peaks in the HPLC chromatograms were determined by MALDI analysis of collected fractions. We found that when the DCL compositions were analysed directly using MALDI-MS, the mass intensities correlated well with the concentrations determined by HPLC, at least for the narrow range of masses (ca. 950 – 1400 m/z) where the $[M+Na]^+$ ions for CDs were observed (ESI Section S5). Both HPLC-ELS and MALDI-MS were, therefore, deemed suitable for

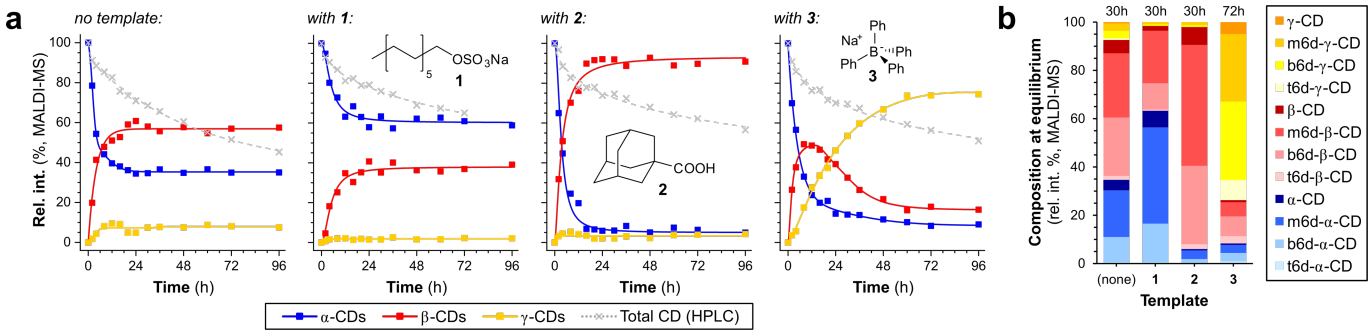

Fig. 3 Templated dynamic libraries of 6-deoxyglucopyranose-containing CDs. (a) Relative intensities (MALDI-MS) of CDs (grouped into α, β and γ-CDs) observed over time in libraries started from **m6d-α-CD** in presence of templates **1**, **2** or **3**. The total CD concentration (as quantified by HPLC-ELS) is illustrated with a grey dashed line (% by weight out of all α-glucans). (b) CD distribution observed at pseudo-equilibrium (in % according to rel. int., MALDI-MS). Reaction conditions as in Fig. 1b.

relative quantification of the components in these mixtures of modified CDs.

It was found that the two DCLs converged on identical compositions within approximately 1 day (ESI Section 3.1). Figure 2a shows the identical HPLC chromatograms obtained after 30 hours of reaction and Figure 2b shows how the concentrations of the different CDs in the two mixtures changed over time as they move towards the same product distribution. As a further test of the dynamic nature of the system, two different pre-formed libraries, either started from **m6d-α-CD** or from **m6d-β-CD**, were mixed together after 8 hours initial reaction. Within 40 hours subsequent equilibration time, this resulted in identical composition to a DCL started directly from a 1:1 mixture of **m6d-α-CD** and **m6d-β-CD** (ESI Section 3.2).

Next, we investigated the addition of templates to alter the composition of these DCLs of modified CDs. To that end, we started a set of DCLs by exposing **m6d-α-CD** (5 mg/mL) to CGTase in the presence of different templates (5 mM) under conditions as described above, and we monitored the evolution of the DCLs using a combination of HPLC-ELS and MALDI-MS (Figure 3). Sodium dodecyl sulfate (**1**), 1-adamantane carboxylic acid (**2**) and sodium tetraphenylborate (**3**) were employed as templates, as they have been shown to favour the formation of α, β and γ-CD, respectively, in dynamic libraries of unmodified CDs.[6]

Templates **1**, **2** and **3** were found to favour the formation of both modified and unmodified α-CDs, β-CDs and γ-CDs, respectively, compared to an untemplated library. The equilibrium compositions were reached within 24–36 hours for the reference DCL and the DCLs templated with **1** and **2**, while the DCL templated with **3** first produced β-CDs (similar to the untemplated library) before slowly building up γ-CDs to reach an equilibrium distribution after 3–4 days (Figure 3). Slow production of γ-CD, also in presence of **3**, has been observed in previously reported libraries of unmodified CDs using the same enzyme.[5] The templates chosen here were not designed to be able to distinguish between modified and unmodified CDs and the preferential amplification of CDs with a defined number of 6-deoxyglucopyranose units was not observed. Figure 3a shows how the relative distributions of α-CDs, β-CDs and γ-CDs evolved over time to give stable distributions, while Figure 3b shows the relative distributions of the different modified CDs once equilibrium was reached. Interestingly, the proportion of 6-deoxyglucopyranose units found in CDs was found to increase with longer reaction times, while the proportion of 6-deoxyglucopyranose units amongst the linear products decreased. This tendency was judged to result from a slower rate of hydrolysis of modified CDs compared to natural CDs, meaning that the modified CDs remained kinetically trapped in the CD subsystem for longer, rather than from selective or stronger interactions between the templates and the modified CDs (ESI Section 6).

Finally, we investigated whether CGTase can accept CDs with other modifications at the 6-position as substrates. Commercially available mono-6-azido-6-deoxy-glucopyranose (**m6N$_3$-β-CD**) and mono-6-deoxy-6-iodo-glucopyranose (**m6I-β-CD**) were tested as substrates. It was found that exposing these modified β-CDs to CGTase also resulted in complex dynamic mixtures of α-, β- and γ-CDs with 0, 1 or 2 modified glucopyranose units. These DCLs also responded to the presence of templates **1**, **2**, **3** in a similar manner to the DCLs formed from 6-deoxy-CDs (Figures 4a,b and S26-S27 in the ESI). In the MALDI-MS analysis of these DCLs, the relative peak intensities for the modified CDs compared to the unmodified CDs were smaller when compared with the DCLs of 6-deoxy-CDs (compare Figures 4a,b with Figure 1b). Also, t6I-CDs and t6N$_3$-CDs were not observed at all. This indicates that glucopyranose units with larger substituents are less easily incorporated into CDs compared with 6-deoxy-glucopyranose units or that these modifications dramatically decrease the ionisability of the CDs. Meanwhile, it was possible to form a DCL by combining a 1:1 (w/w) mixture of **m6d-β-CD** and **m6I-β-CD** (total concentration 5 mg/mL) in the presence of CGTase. This resulted in a dynamic mixture that included CDs containing both 6-deoxy- and 6-deoxy-6-iodo-glucopyranoses in the same macrocycle (Figure 4c). This library also adapted to the addition of templates **1** – **3** (Figure S28, ESI), which shows that dynamic systems of modified CDs can be used to obtain CDs with several different modifications.

Conclusion

We have shown it is possible to generate enzyme-mediated dynamic combinatorial libraries of modified (unnatural) CDs.

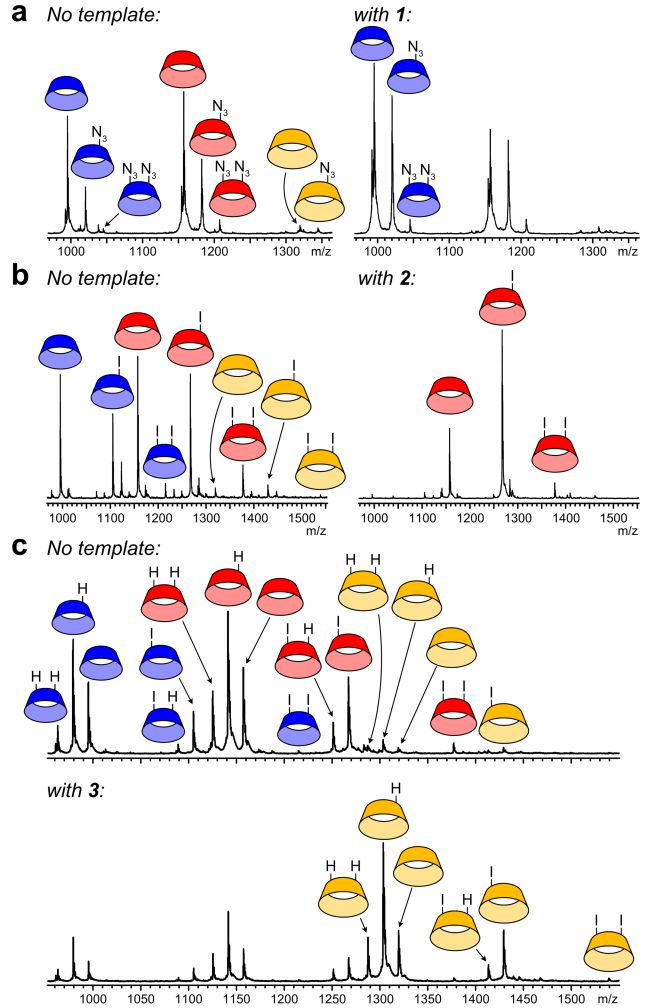

Fig. 4 Dynamic mixtures of different 6-modified CDs. Spectra (MALDI-MS) of mixtures obtained when starting libraries from (a) **m6N$_3$-β-CD** in absence or presence of **1**; or (b) **m6I-β-CD** in the absence or presence of **2**; or (c) a 1:1 (w/w) combination of **m6d-β-CD** and **m6I-β-CD** in the absence or presence of **3**. Only amplified species are highlighted in templated libraries. Conditions as in Fig. 1b.

Using two novel CDs, **m6d-α-CD** and **m6d-β-CD**, we have demonstrated how kinetically trapped dynamic systems of modified CDs operate under *pseudo*-thermodynamic control, wherein the CD distribution reflects their relative stabilities and can be manipulated via interaction with a template. DCLs formed from **m6N$_3$-β-CD** and **m6I-β-CD** highlight the promiscuity of CGTase. Future work will consider the design of templates that can select not only the CD size, but also the number, position, and functionality of the modified glucopyranose units. Nevertheless, this work represents an important first step towards the templated dynamic enzymatic synthesis of selectively modified CDs.

Conflicts of interest

There are no conflicts to declare.

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