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Tuning the outcome of enzyme-mediated dynamic cyclodextrin libraries to enhance template effects

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Abstract: Enzyme-mediated dynamic combinatorial chemistry combines the concept of thermodynamically controlled covalent self-assembly with the inherent biological relevance of enzymatic transformations. We explore a system of interconverting cyclodextrins wherein the glycosidic linkage is rendered dynamic by the action of cyclodextrin glucanotransferase (CGTase). We report that external factors, such as pH, temperature, solvent, and salinity can be modulated to influence the composition of the dynamic cyclodextrin library. Dynamic libraries of cyclodextrins (CDs) could be obtained in wide ranges of pH (5.0–9.0), temperature (5–37 °C), and salinity (up to 7.5 M NaNO3), and with high organic solvent content (50 % by volume of ethanol), showing that enzyme-mediated dynamic systems can be robust and not limited to physiological conditions. Furthermore, we demonstrate how strategic choice of reaction conditions can enhance template effects, in this case, to achieve highly selective production of α-CD, an otherwise challenging target due to competition from the structurally similar β-CD.

Introduction

Dynamic combinatorial chemistry has evolved into a powerful tool to explore molecular self-assembly and systems chemistry behavior, and to identify macroyclic hosts for small molecules or ligands for biomacromolecules.[1] Dynamic covalent reactions have been exploited to develop smart materials,[2] to generate self-replicating systems,[3] and have enabled access to complicated macrocycles such as (hemi)cucurbit[n]urils, biotin[6]urils, cryptates and larger nano-rings.[4] Significant effort has been exerted to develop synthetic dynamic systems that mimic biological dynamic systems. Dynamic combinatorial libraries (DCLs) using biomimetic building blocks[5] have been explored and “biocompatible” reversible chemistries that work in water under biologically relevant conditions have been targeted, for example, by using thiol exchange at neutral pH,[6] or by developing catalysts for hydrazine and oxime exchange at neutral pH.[7] Enzymatic transformations typically take place in water at moderate temperature and close to neutral pH, and act on inherently biologically relevant building blocks (i.e. biomonomers such as amino acids, nucleotides, or carbohydrates). Despite the promises of applying enzymes to make “biocompatible” or biomimetic dynamic systems, less than a handful of enzyme-enabled dynamic systems have been reported to date.

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Supporting information for this article is given via a link at the end of the document.

The group of Venton reported the first example of an enzyme-mediated DCL, when they employed trypsin to make a small dynamic library of oligopeptides.[8] Brown and co-workers reported the use of a sialic acid aldolase to produce libraries of sialic acid analogues,[9] and Flitsch and co-workers demonstrated protease enabled amine acyl exchange on gold surfaces.[10] Most recently the Ulijn lab have developed protease-enabled dynamic peptide libraries employed towards the formation of supramolecular nanostructures.[11]

We recently reported how the enzyme cyclodextrin glucanotransferase (CGTase) can be used to afford a dynamic system of interconverting linear and cyclic oligomers of glucose connected by α(1–4) glycosidic bonds (Figure 1a).[12] CGTase catalyses two reactions: both reversible inter- and intramolecular transglycosylations to yield linear or cyclic α-glucans (cyclodextrins), respectively; and slow hydrolysis (Figure 1b).

The dynamic system consists primarily of the three macrocyclic native cyclodextrins (CDs) α-CD, β-CD, and γ-CD (with 6, 7, and 8 glucopyranose units, respectively) which are formed in an approximately 35/60/5 ratio. Due to CGTase’s kinetic preference for macrocyclisation, the CDs are formed rapidly in a kinetically trapped subsystem, which behaves as a transient DCL over which there is pseudo thermodynamic control. Slow background hydrolysis means that eventually, over many days, all α-glucans in the system are converted to glucose.[12]

CDs are one of the most important and well-studied macrocyclic structures in supramolecular chemistry,[13] with applications in, amongst others, the food, cosmetics and pharmaceutical industries where their role as water soluble hosts for hydrophobic guests is exploited.[14] We showed that addition of templates (guests) that bind selectively to specific CDs can alter the CD distribution in CGTase-mediated cyclodextrin DCLs to produce each of the three CDs with 89 – 99 % selectivity. Furthermore, we demonstrated that templates that bind to even larger CDs, e.g. β-CD or γ-CD with 9 and 10 glucose units, respectively, can be employed to produce these large-ring CDs with hitherto unmatched selectivity.[12a]

The glycosidic bond can be made dynamic by the action of CGTase under mild conditions (pH 7.5 at room temperature), but during our initial screening for optimal conditions for the enzymatic reactions, we noticed that CGTase was remarkably resilient to changes in conditions such as pH, salinity, solvent composition and presence of templates. While the influence of some of these parameters on CGTase activity, CD yield and selectivity in kinetically controlled regimes relevant for industrial production has been investigated,[15] we were interested to explore how such (non-template) external factors would affect the dynamicity and equilibrium distribution of CDs in our dynamic system. We present here how tuning of pH, temperature, building block concentration, solvent composition and salinity can be employed to control the equilibrium CD distribution in CGTase-mediated DCLs and enhance template effects.
while initially highly active at 50 °C, CGTase was rendered effectively inactive after 1 day at such a high temperature, both at pH 5.5 and pH 7.5 (Sections S2 and S3 in the Supporting Information). To establish an enzyme-mediated DCL we required long-term enzyme stability, at least long enough that a thermodynamically-controlled distribution of library members can form. Fortunately CGTase activity could be retained lowering the temperature to 37 °C (Section S3 in the Supporting Information). Furthermore we have recently shown that it is possible to establish a dynamic system of linear and cyclic α-glucans at 25 °C that stays dynamic for at least 31 days.\textsuperscript{12a}

Figure 2a shows a representative chromatogram of the α-glucan mixture formed by CGTase action on G6 at 37 °C after 60 minutes. Linear α-glucans G1–G8 (seen as split peaks due to pairs of anomers) and, α-, β-, and γ-CD are clearly identifiable. At 37 °C a steady distribution of the different CDs is observed after approximately 30 minutes, when a pseudo thermodynamic equilibrium distribution of cyclodextrins is obtained (Figure 2c). As the temperature was reduced, the approach to equilibrium was slower, but a steady CD distribution was achieved within two hours even at 5 °C. By analysing the rate of consumption of hydrolysable glucose monomer units (all glucose units except the reducing ends of linear library members) and the time taken to reach the half maximum CD yield, we estimated apparent rate constants for CD formation (k\text{co}) and the hydrolysis side reaction (k\text{h}) (Section S4.1 and Figures S4–S5 in the Supporting Information). Both k\text{co} and k\text{h} decreased upon lowering the temperature (Figure S6 in the Supporting Information) and consequently temperature variation had very little influence upon the total CD yield (Figure 2 grey lines).

We noticed, however, a small but significant change in the equilibrium CD distribution as a function of temperature (Figure 2b). The relative α-CD concentration at equilibrium increased with higher reaction temperature, while the relative yield of both β-CD and γ-CD decreased. As there is an inherently higher entropic penalty for the formation of larger CDs, this result is in line with the notion that entropic factors play a larger role in the overall free energy of the system at higher temperatures. Control over reaction temperature within the determined dynamic range (5 °C to 37 °C) could thus be exploited to target specific CD products.

The influence of pH
To gain an overview of the influence of pH on enzyme activity, as well as the dynamics and the equilibrium CD distribution in the CGTase-mediated DCLs, we set up a series of libraries started from maltohexaose (G6) at pH 4.0 – 9.0 (10 mg/mL, 22 °C, 50 mM buffer, either sodium citrate, sodium phosphate or tris(hydroxymethyl)aminomethane (tris) (Figure S7 and S8 in the Supporting Information). We monitored the formation of cyclic and linear α-glucans over at least 60 hours (Figures S7 and S8 in the Supporting Information).

Figures 3a and 3b compare the extent of CD formation after 20 minutes and the degree of hydrolysis from 24 hours to 60 hours in libraries prepared at different pH, as a measure of initial and continuing enzyme activity, respectively. At pH 4.0 and 4.5, CGTase became inactive within less than one day and an

Results and Discussion
The influence of temperature on cyclodextrin DCLs
To explore the influence of temperature on the distribution of products in cyclodextrin DCLs and the overall CD yield, CGTase-mediated dynamic cyclodextrin systems were prepared at 5 °C, 22 °C and 37 °C. CGTase from Bacillus macerans (50 μL of a ca. 12 mg/mL stock solution per 1 mL of reaction mixture) was allowed to act on maltohexaose (G6) (10 mg/mL) in sodium phosphate buffer (50 mM) at pH 7.5 and the formation and disappearance of the various cyclic and linear α-glucans was monitored as the dynamic system evolved over time. (Figure 2a and Figure S3 in the Supporting Information). Analysis was achieved using high performance liquid chromatography with evaporative light scattering detection (HPLC-ELS), which enables detection of the otherwise chromophore- and fluorophore-less oligosaccharides.

The industrial production of CDs via the action of CGTase is typically performed at relatively high temperature (50 – 70 °C)\textsuperscript{19} and indeed an optimal reaction temperature of 60 °C has been reported.\textsuperscript{19c} In preliminary experiments, however, we found that

![Figure 1](Image)

**Figure 1.** (a) Cyclodextrin glucanotransferase (CGTase) acts on α-glucans to generate dynamic combinatorial libraries (DCLs) of interconverting cyclodextrins. Changing the pH, temperature, concentration, solvent composition and salinity allows control over the outcome of the enzyme-mediated DCL. (b) The reactions catalysed by CGTase. Transglycosylation is reversible and includes both an intramolecular reaction that leads to CD formation (macrocyclisation) and an intermolecular reaction that can shorten and elongate linear α-glucans (scrambling). Hydrolysis leads to the formation of a new reducing-end glucose unit (highlighted in red), which cannot be reincorporated into a macrocycle, n, m = [0, 1, 2, ...] x, y, z = [1, 2, 3, ...].
equilibrium distribution of CDs was never reached. The reactions conducted at pH 5-8 reached a steady CD distribution within ca. 1 to 1½ hours, while the libraries at pH 8.5 and pH 9.0, required up to ca. 3 and 6 hours, respectively. It should be noted that significant build-up of byproducts occurred in reactions in tris buffer after several days due to a slow reaction with the buffer itself (Section S5.1 in the Supporting Information).

The equilibrium distributions of α-, β-, and γ-CD at different pH values are plotted in Figure 3c. The ratio of CDs is relatively constant over the entire pH range 5.0 – 9.0, which is consistent with the expectation that pH should not influence relative CD stability. A slight increase in the α-CD content was seen upon switching from citrate buffer to phosphate buffer, which might be explained by the higher ionic strength of phosphate buffers compared to citrate buffers (salt effects are discussed below). Overall, we found that the remarkable pH stability of CGTase means that we can prepare truly dynamic systems of CDs over a wide range of pH (5.0 to 9.0). This versatility can be an advantage because it broadens both the selection of templates that can be employed, and the different chemistries that could be explored alongside CGTase action in more complex dynamic systems.

Concentration effects

The equilibrium distribution of oligomeric species in DCLs is dependent on the building block concentration.\(^{14,16}\) To examine the effect of total sugar concentration on the equilibrium CD distribution and total CD yields, we set up and monitored the action of CGTase on 1 – 40 mg/mL of G6 in phosphate buffer at pH 7.5 (Figures S11 and S12 in the Supporting Information). The equilibrium CD distributions as a function of starting concentration of G6 are shown in Figure 4a. As expected, the proportion of larger CDs (β- and γ-CD) goes up as the total sugar concentration increases, since larger macrocycles are favoured at higher building block concentrations.

Figure 4a also shows the maximum CD yield obtained from different starting concentrations of G6. The maximum CD yield was found to increase upon increasing the concentration of G6 from 1 to 5 mg/mL but decrease upon moving to even higher G6 concentrations. As the concentration increases the rate of intermolecular transglycosylation (scrambling) will increase relative to both the rates of hydrolysis and intramolecular transglycosylation (macrocyclization). A relatively slower rate of hydrolysis will favour high CD yields, but a relatively slower rate of macrocyclisation will lead to low CD yields. It appears that
a glucan concentration of approximately 5 mg/mL is where a balance between hydrolysis and excessive intermolecular transglycosylation leads to the highest CD yield.

The relatively higher rate of hydrolysis compared to intermolecular transglycosylation at low glucan concentrations meant that the lifetime of the dynamic system decreased dramatically with decreasing concentration following an apparently exponential order in relation to G6 starting concentration (Figure 4b). Here, we defined the lifetime of the system as the half-life ($t_\text{h}$) of the hydrolysis reaction (Section S6.1 in the Supporting Information). At $t_\text{h}$, half of the glucose units in the dynamic system have been converted to reducing ends, and can no longer be used to form CDs. This value is therefore directly related to the lifetime of the transient DCL of CDs. While 5 mg/mL starting concentration of G6 is optimal to give high CD yields, the lifetime of the transient DCL is rather short. We suggest an optimal glucan concentration of ca. 10 – 20 mg/mL for these CGTase-mediated dynamic cyclodextrin systems.

The influence of ethanol as organic co-solvent

It has been reported that addition of polar organic solvents can enhance the yield and selectivity of CGTase-catalysed CD production from starch.\textsuperscript{[17,18]} However, whether these effects are due to solvent influences on CGTase activity, substrate selectivity, formation of inclusion complexes or kinetics has remained unclear, in part, because many studies have been based on single timepoint analysis of reactions without taking into consideration the complexity and dynamic nature of the CGTase-mediated cyclodextrin system. To explore the effect of an organic co-solvent on our dynamic system of CDs, we set up a series of reactions starting from G6 (10 mg/mL) in phosphate buffer (50 mM, pH 7.5)

![Figure 3. pH screening. (a) Activity of CGTase at different pH and in different buffers quantified as the relative CD formation over the first 20 minutes of reaction time. (b) Activity of CGTase after 24 hours at different pH and in different buffers quantified as the amount of hydrolysis over the following 36 hours. (c) Equilibrium CD distribution and maximum CD yield as a function of buffer and pH. Conditions: G6 (10 mg/mL) in indicated buffer (50 mM) at indicated pH treated with CGTase at room temperature.]

![Figure 4. Concentration effects. (a) Equilibrium CD distribution and maximum CD yield as a function of G6 starting concentration. (b) Lifetimes of dynamic systems as a function of G6 starting concentration. The lifetime is defined as the half-life of hydrolysis assuming pseudo-first order kinetics. Conditions: G6 at indicated concentration in sodium phosphate buffer (50 mM, pH 7.5) treated with CGTase at room temperature.]

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Figure 5. Ethanol as co-solvent. (a) CD distribution at equilibrium and highest CD yields when CGTase acts on G6 in the presence of different amounts of ethanol as co-solvent. The reactions with 40 and 50 % ethanol by volume did not reach equilibrium within 72 hours. (b) Change in half-lives (\(t_{1/2}\)) of the hydrolysis and CD formation reactions catalysed by CGTase as a function of the ethanol content (see ESI for details). Conditions: G6 in sodium phosphate buffer (25 mM, pH 7.5) treated with CGTase at room temperature.

with 0 – 50 % by volume of ethanol and monitored the total CD yield and CD distribution over a period of 72 hours (Figures S14 and S15 in the Supporting Information).

In all cases, the addition of ethanol had a stabilising effect on \(\alpha\)-CD (Figure 5a), which is consistent with previously reported higher \(\alpha\)-CD yields observed in the presence of ethanol.\(^{[15]}\) The equilibrium CD distribution shifted dramatically from ca. 35% \(\alpha\)-CD in aqueous buffer to ca. 80% \(\alpha\)-CD even in the presence of only 10 % ethanol by volume (Figure 5a). The sharp change in composition with any amount of ethanol suggests an actual binding event rather than a solvent effect (i.e. change in solvent polarity). \(\alpha\)-CD has a reported weak binding affinity (ca. \(K_a = 10\) \(\text{M}^{-1}\)) towards ethanol in water, with no reported affinities for \(\beta\)- or \(\gamma\)-CD towards ethanol.\(^{[15,18]}\) However, the continued presence of 20% \(\beta\)-CD in the DCL, despite such high concentrations of ethanol, indicates that a weak interaction must exist between \(\beta\)-CD and ethanol. Using the DCLfit software, developed by Otto to extract binding constants directly from equilibrium product distributions in templated DCLs,\(^{[20]}\) we estimate that \(\beta\)-CD binds ethanol roughly 6 – 7-fold weaker than \(\alpha\)-CD (Section S7.1 in the Supporting Information).

In our experiments, and consistent with previous studies, the addition of ethanol led to a small increase in the maximum CD yield obtained.\(^{[18]}\) Previous reports hypothesised that this yield increase might be due to a relatively reduced rate of hydrolysis, compared to CD formation.\(^{[17]}\) However, detailed kinetics analysis (Section S7.2 and Figures S17 and S18 in the Supporting Information) revealed an exponential decay in the rates of both hydrolysis and CD formation reactions in the presence of ethanol, as evidenced by the linear correlation between the logarithm of the reaction half-lives and the ethanol content (Figure 5b). This result is consistent with the observation that our dynamic system develops increasingly more slowly as the ethanol content is increased (time to equilibrium is <1 hour in aqueous solution, several hours with 20% ethanol, 1 day with 30% ethanol and >3 days with 40-50% ethanol). Our data shows ethanol retards both the CD formation and hydrolysis rates to a similar extent. We propose that the higher maximum CD yield observed in the presence of ethanol is in fact a consequence of ethanol binding to \(\alpha\)-CD after it forms and thus ‘protecting’ this substrate from the action of CGTase by host-guest complex formation.

The influence of high salt content

Since the hydrophobic effect is thought to be the main driving force for host-guest interactions of CDs, and the hydrophobic effect can be enhanced by addition of chaotropic salts, we were interested to explore the influence of high salt concentrations on our dynamic CD system. Recent reports have indicated that strong interactions can occur between CDs and chaotropic anions,\(^{[21]}\) and NaNO\(_3\) has frequently been used in supramolecular systems and DCLs in order to increase hydrophobic effects.\(^{[22]}\) We thus prepared and closely monitored a series of CGTase-mediated dynamic CD systems starting from G6 (10 mg/mL) in phosphate buffer (40 mM at pH 7.5) at room temperature in the presence of 0 – 7.5 M NaNO\(_3\).

Remarkably, the enzyme retained its activity at these very high salt concentrations, reaching a steady equilibrium distribution of CDs within 1-3 hours. However, the relative concentrations of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-CD obtained at equilibrium was strongly influenced by the presence of salt (Figure 6). A gradual increase in the relative concentration of \(\alpha\)-CD from 34% to 79% in the presence of 7.5 M NaNO\(_3\) was seen accompanied by a concomitant decrease in \(\beta\)-CD and \(\gamma\)-CD concentration. Although a 7.5 M NaNO\(_3\) concentration is equivalent to ca. 46 % by weight in the solvent, and is equivalent to just 5.5 water molecules per NaNO\(_3\) moiety, only a modest change in the equilibration time was observed, with the system needing closer to 3 hours to reach equilibrium in the presence of higher concentrations of NaNO\(_3\) (Figures S19 and S20 in the Supporting Information). Salt-induced changes in dynamic systems have been observed before,\(^{[23]}\) but not in a system of uncharged macrocycles such as CDs. Further experiments are underway to determine whether the dynamic system reacts differently to kosmotropic and chaotropic salts.
Enhanced template-directed selective synthesis of α-CD

In a previous study exploring the use of templates to selectively synthesise different CDs in our CGTase-mediated DCLs, we could obtain β-CD with >99% selectivity by adding 1-adamantane carboxylic acid, and γ-CD with >99% selectivity by adding tetraphenylborate, but the highest α-CD selectivity we managed to obtain was 89% using sodium dodecyl sulfate (SDS) as the template. In our study, α-CD was shown to bind 2:1 to SDS with two high affinities (Kd of (1.9 ± 0.5)·10^4 M^-1 and Kd of (2.3 ± 0.4)·10^4 M^-1), the low selectivity was due to a competing strong interaction between SDS and β-CD (Kd of (1.6 ± 0.4)·10^4 M^-1). Seeking to improve this result, we sought now to apply our new knowledge of reaction condition influences on the thermodynamics of our cyclodextrin DCL, to enhance the template-directed selective synthesis of α-CD.

The entropic penalty (ΔS) for the formation of a 2:1 complex is larger than for the formation of a similar 1:1 complex. Since the magnitude of the entropic contribution to binding free energy change depends on temperature (ΔG = ΔH – T·ΔS), it seemed plausible that the 2:1 (α-CD):SDS complex would be relativelyfavoured over the similar 1:1 CD:SDS complex at lower temperatures, leading to enhanced α-CD production. (Figure 7a).

We set up and monitored a series of CGTase reactions starting from G6 (10 mg/mL) at pH 7.5 (50 mM sodium phosphate buffer) with 5 mM SDS (the optimal concentration to favour 2:1 binding determined in our previous study) in a range of temperatures (5, 22, and 37 °C) (Figures S21 and S22 in the Supporting Information). The equilibrium CD distribution as a function of temperature is shown in Figure 7b. As anticipated, α-CD selectivity increased upon lowering the temperature. In fact, at 5 °C α-CD was formed with 99 % selectivity and in high yield, which highlights how strategic selection of reaction conditions in these CGTase-mediated DCLs can be exploited to enhance template effects and control the outcome of an enzyme-mediated process.

Conclusions

In conclusion, we have demonstrated how turning the dials of pH, temperature, concentration and solvent polarity, in the form of adding organic solvents or inorganic salts to the aqueous reaction, allows strategic control over the outcome of the CGTase-mediated dynamic cyclodextrin system. These non-template effects can be combined with templates for enhanced control of selectivity. Here we demonstrated how the system can be manipulated so that CGTase generates α-CD as the only significant CD product. Furthermore, the robustness of this system shows that enzyme-mediated dynamic combinatorial chemistry need not be confined to narrow ranges of pH, temperature and solvent compositions. We hope that this study will encourage the further exploration of different reversible enzymatic transformations for the generation of dynamic chemical systems.

![Figure 6](Image)

**Figure 6.** Equilibrium CD distribution as a function of NaN3 concentration. Conditions: G6 (10 mg/mL) in sodium phosphate buffer (40 mM, pH 7.5) treated with CGTase at room temperature in the presence of the indicated amount of sodium nitrate.

![Figure 7](Image)

**Figure 7.** Enhancing α-CD selectivity. (a) Model of the 1:1 complexes and 2:1 complex formed in an enzyme-mediated DCL of CDs when SDS is employed as a template. (b) Equilibrium CD distribution obtained in the presence of SDS as a function of temperature. Conditions: G6 (10 mg/mL) in sodium phosphate buffer (50 mM) at pH 7.5 treated with CGTase at indicated temperature in presence of SDS (5 mM). Results at 25 °C from previous study are marked with asterisks (*)
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Keywords: Cyclodextrins • Dynamic Combinatorial Chemistry • Enzymes • Host-guest systems • Supramolecular Chemistry


Tuning in to enzyme-mediated dynamic systems. CGTase-mediated dynamic combinatorial libraries of cyclodextrins can be established at wide ranges of pH, temperature, solvent composition and salinity. Control of these conditions determines which cyclodextrins are preferentially produced. Combining the influence of reaction conditions with known template effects allows us to produce α-CD with remarkably high selectivity.