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pH-responsive templates modulate the dynamic enzymatic synthesis of cyclodextrins

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Product selection in the dynamic enzymatic synthesis of cyclodextrins can be controlled by changing the pH. Using cyclodextrin glucanotransferase to make labile the glycosidic linkages in cyclodextrins (CDs), we generate a dynamic combinatorial library of interconverting linear and cyclic α-1,4-glucans. Templates can be employed to favour the selective production of specific CDs and, herein, we show that by using ionisable templates, the synthesis of α-CD or β-CD can be favoured by simply changing the pH. Using 4-nitrophenol as the template, β-CD is the preferred product at low pH, while α-CD is the preferred product at high pH. Furthermore, a new methodology is described for the simulation of product distributions in dynamic combinatorial libraries with ionisable templates at any given pH.

In biological systems, the pH regulation of enzyme activity is critical to cellular functions such as metabolism and signal transduction.[1] A mild shift in pH away from the optimum of an enzyme typically results in a reversible (partial) deactivation, which down-regulates that enzyme's activity, and associated biochemical pathways, until the pH optimum is re-established.[2] In biocatalysis, stimuli-responsive modulation of individual enzyme activity in multi-enzyme cascades could give remotely controlled selection of specific reaction pathways and tuneable product outcomes.[3–5] Light and temperature control of enzyme activity has been achieved via bioconjugation of enzymes with light and thermo-responsive molecules and polymers.[6–7] pH modulation of enzyme activity has been described using reversible pH-controlled encapsulation of enzymes inside DNA nanocages[8] and lipid vesicles.[9] Herein, we describe the use of pH to control product selection in an enzymatic synthesis. A pH change is not employed to turn on/off enzyme activity. Instead, the pH change switches on/off a templating effect. We describe how a simple change of pH can be used to alter product selectivity in the dynamic enzymatic synthesis of cyclodextrins.

Cyclodextrins (macrocyclic α-1,4-linked glucans) are highly important molecular hosts for hydrophobic guests that are extensively employed in the food, cosmetics, and pharmaceutical industries.[10–12] They are produced enzymatically on an industrial scale from starch in a process wherein the enzyme cyclodextrin glucotransferase (CGTase) generates a mixture of mostly α, β, and γ-CD, with 6, 7, and 8 glucopyranose units, respectively.[13] Separation of these products is challenging and different approaches have been explored to favour selective synthesis of each CD, including CGTases from different sources, modified CGTases, use of complexing agents, and optimisation of reaction conditions, such as temperature, concentration and reaction time. Change of pH modulates the level of CGTase activity, but alone has little influence on product selection.[14] We have previously shown how light can be used to control the selective synthesis of CDs using a photoswitchable template.[15–16] Here, we instead employ a pH-responsive template.

Dynamic combinatorial chemistry enables the synthesis under thermodynamic control of mixtures of products formed from simple building blocks linked together using reversible (covalent) bonds.[17–18] The distribution of products formed in a dynamic combinatorial library (DCL) can be perturbed by interaction with a template, or due to physical or chemical stimuli. In enzyme-mediated dynamic combinatorial chemistry,[19–24] covalent bonds that are usually considered static can be rendered labile, enabling dynamic mixtures of biooligomers to form. This has given rise to the discovery of new adaptive biomaterials[22] and the development of a novel template-driven approach to the synthesis of cyclodextrins.[19–21]

We have previously described how the action of cyclodextrin glucanotransferase (CGTase) on α-1,4-glucans leads to a dynamic combinatorial library (DCL) of linear and cyclic α-1,4-glucans.[19,20] CGTase catalyses reversible inter- and intramolecular transglycosylation and slow hydrolysis of α-1,4-

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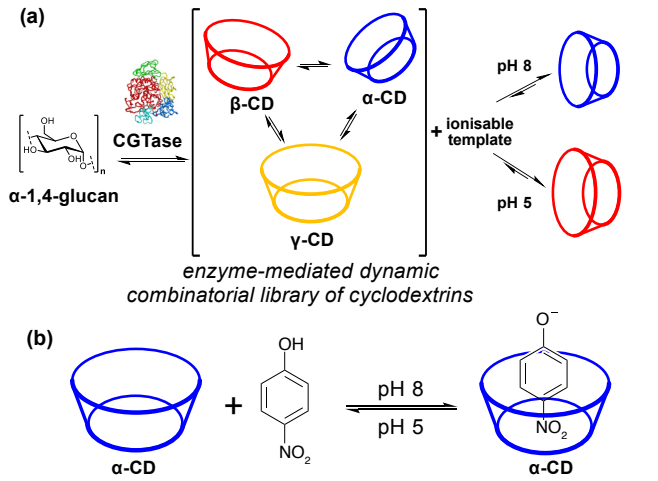

Figure 1 (a) pH-controlled selective enzymatic synthesis of cyclodextrins using ionisable templates to direct the outcome of a CGTase-mediated dynamic combinatorial library of cyclodextrins. (b) The preferential binding of 4-nitrophenolate to α-CD is exploited in this work.

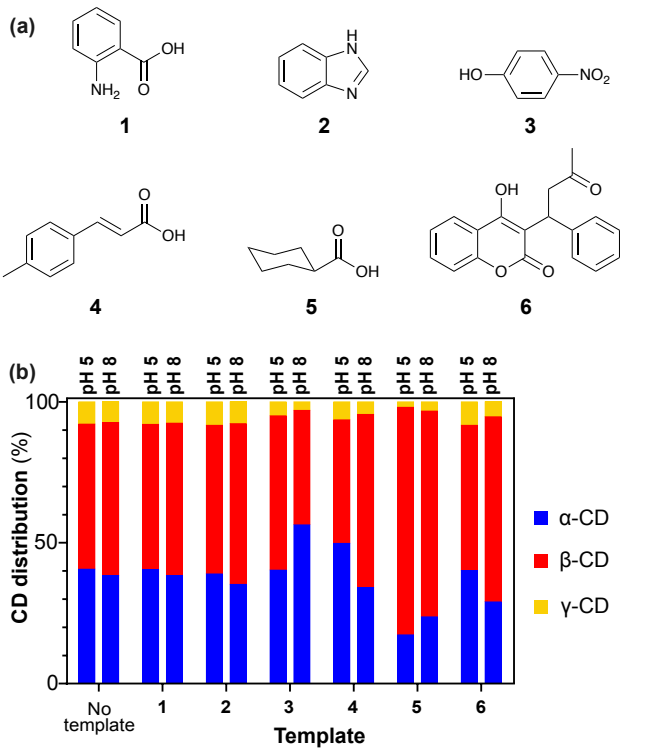

Figure 2 Screening of different ionisable template for their pH-sensitive impact on CGTase-mediated cyclodextrin DCLs. (a) Ionisable templates tested: 2-aminobenzoic acid (**1**), benzimidazole (**2**), 4-nitrophenol (**3**), 4-methylcinnamic acid (**4**), cyclohexane carboxylic acid (**5**) and warfarin (**6**). (b) CD distributions obtained in DCLs started from α-CD (2 mg/mL) templated with **1**–**6** (2 mM) at pH 5 and pH 8.

glucans within the pH range 5-8.[14] The resulting DCL consists mainly of the three CDs; α-, β- and γ-CD, but these are kinetically trapped, and due to the slow hydrolysis, the thermodynamic end-product of the enzymatic reaction is glucose.[19] Nevertheless, during the lifetime of the CDs (approximately 1 day), the CD sub-systems exist under *pseudo*-thermodynamic control, that is, the distribution of CDs formed reflects their intrinsic relative stabilities and any stabilising external influences, such as complex formation with added templates. We have previously reported the template-directed dynamic enzymatic synthesis of α-, β- and γ-CD with 99% selectivity using sodium dodecylsulfate, adamantane carboxylic acid and tetraphenyl borate, respectively, as templates.[19–20]

It is well-established that complex formation between CDs and ionisable guests in aqueous solution is affected by pH.[25–28] Preferential binding of the neutral form is typically observed, but, conversely, phenolates bind more strongly than the corresponding phenols.[27,28] The affinity of ionisable guests for the CD cavity at a given pH is dependent upon the relative presence of the neutral and ionised forms. We have previously shown how the apparent binding affinity can be modelled as a function of pH.[30,31] Considering that the binding affinity is linked to the pH, the template-directed enzyme-catalysed synthesis of CDs should be controllable via ionisation of the template (Fig. 1). Here, we demonstrate how pH changes can direct the preferential enzymatic synthesis of either α-CD or β-CD by use of an ionisable template.

We first needed to identify ionisable templates with pK_a values of ca. 4–8, which would ensure a significant shift in the ionisation state of the template across the working range of the CGTase (pH 5–8), with reported (pH-dependent) affinities for a specific CD, and with at least mM solubility over the required pH range. Templates **1**–**6** were selected for this study (Fig. 2a). A commercially available CGTase stock solution (50 μl/mL) was added to a solution of α-CD (2 mg mL^{-1}) in the presence of one of the six templates (**1**–**6**) (2 mM) in a mixed buffer of acetate and phosphate (50 mM) at pH 5 and at pH 8. This buffer, containing only small kosmotropic anions, was carefully chosen to minimise the possible effects of buffer variation on the CD formation. We have recently seen how weak binding by chaotropic anions can subtly affect the product distribution in DCLs of cyclodextrins.[21] Care was also taken to maintain a constant ionic strength (Section S1.3, ESI†). The composition of each DCL was followed over time from 0–5 hours, using high performance liquid chromatography with evaporative light scattering detection (HPLC-ELS). In all cases, the α-CD was rapidly converted to a mixture of mainly α- and β-CD and a little γ-CD, as well as some short linear α-1,4-glucans, that built up over time due to background hydrolysis (Figs. S1, S3, S4, ESI†). The relative amounts of α-, β-, and γ-CD present, once a stable distribution of CDs was first obtained (after 1-2 hours), are shown in Fig. 2b and summarised in Table S1, ESI†.

We found that templates **3**–**6** not only caused a change in the CD distribution compared to the untemplated DCL, but the influence of the templates was pH-dependent. At low pH, **4** caused an amplification of α-CD (41% with no template up to 50% with **4**), but had only little effect at pH 8, suggesting a relatively higher, albeit weak, affinity of the neutral species for α-CD. **5** amplified β-CD both at low and high pH, going from 52% with no template to 81% and 73% at pH 5 and 8, respectively. A slightly stronger amplification at low pH is consistent with the reported higher affinity of the carboxylic acid (K_a = 4.1 × 10^3 M^{-1}), and the not insignificant affinity of the carboxylate (K_a = 2.6

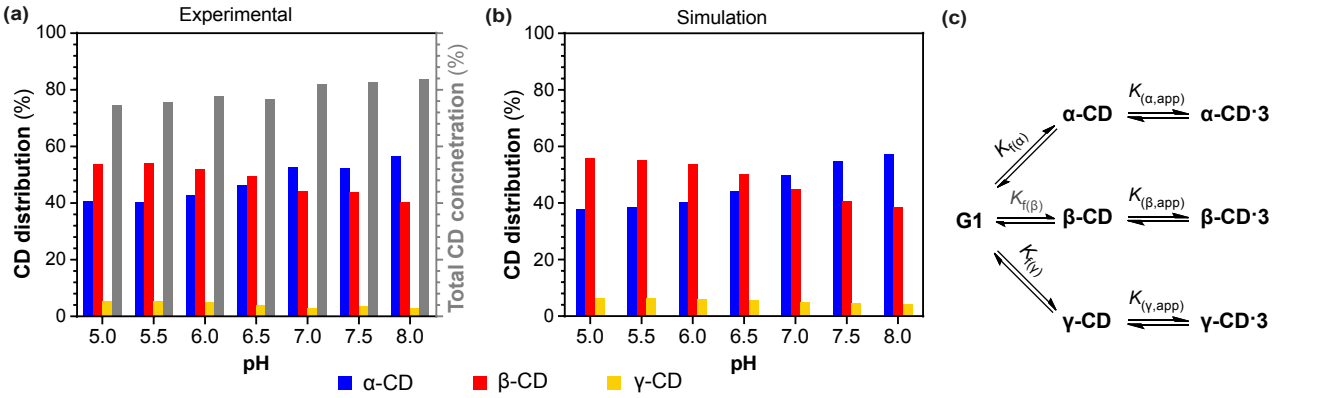

Figure 3 (a) CD distribution and total CD yield at *pseudo*equilibrium for the reaction between α-CD (2 mg/mL) and CGTase with 4-nitrophenol (**3**) (2 mM) in the pH range 5-8. Grey bars show the total CD concentration as a percentage of the total concentration of linear and cyclic α-1,4-glucans present when *pseudo*equilibrium was first reached (b) Simulated CD distributions for experiment reported in (a). (c) Model showing the network of equilibria in the DCL of CDs in the presence of template **3**.

× 10^3 M^{-1}), for β-CD.[10,26] In contrast, it was the ionised version of both 4-nitrophenol **3** and warfarin **6** that caused a change in the CD distribution. At pH 5, neither of these molecules affected the CD distribution, but at pH 8, **3** favoured formation of α-CD (56%), whereas **6** favoured β-CD (66%). The stronger binding of phenolates to α-CD has been explained as resulting from charge delocalisation into the aromatic ring causing stronger dipole-induced dipole interactions with the CD cavity.[28,29] The same argument can be made to explain the apparent higher affinity of the ionised form of **6** for β-CD. 2-Aminobenzoic acid (**1**) and benzimidazole (**2**) had no significant effect on the distribution of the DCL at pH 5 or pH 8 when compared to the untemplated DCL. This is likely due to weak and/or similar affinities for both α- and β-CD in both the neutral and ionised forms.[32]

As 4-nitrophenol (**3**) had the strongest pH-dependent effect on the DCL, this template was chosen for further systematic investigation. α-CD (2 mg/mL) was treated with CGTase in the presence of **3** (2 mM) at 7 different pH values (Fig. S4, ESI†). As a control, the same experiment was performed in the absence of template (Fig. S1, ESI†). The distribution of CDs formed at *pseudo*-equilibrium is shown in Fig 3a and summarised in Table S2 ESI†. These results clearly show how the composition of the DCL is linked to the ionisation degree of the template, since no change was observed in the absence of template. At pH 5, where **3** exists mainly as the neutral species, the CD distribution resembled the untemplated DCL (compare Fig 3a and Fig S2, ESI†). As the pH was raised and the proportion of phenolate increased, the production of α-CD was favoured. A gradual increase in the total CD concentration at the time when a stable distribution of CDs first formed was also observed as the pH was increased (grey bars, Fig 3a). This indicates that there is a relatively reduced rate of hydrolysis, and this trend is consistent with a stronger templating effect at higher pH, as relatively higher concentrations of CDs remain kinetically trapped for longer when there is a binding interaction with a template.[19,20]

An important feature of DCLs operating under thermodynamic control is that they should adapt to changes in their environment in a way that is highly predictable given knowledge of the varying strengths of the supramolecular interactions between library members and templates. The possibility to predict changes to a DCL's distribution upon addition of a template has been explored by Otto and Sanders and the software *DCL Sim* was developed.[33] Here, we have extended this approach and developed a method to predict the concentrations of library members in a DCL in the presence of an ionisable template at any given pH.

Fig. 3c shows a model of the complex network of equilibria in a DCL containing α-, β-, and γ-CD, templated with **3**. To simulate this system at a given pH, we needed values for the formation constant K_f for each CD and for the apparent association constant (K_{app}) for each CD binding to **3** (wherein **3** exists as a pH-dependent mixture of the neutral and ionised species.) In previous work, we have shown how K_{app} for ionisable guests binding to CDs can be modelled as a function of pH, and calculated according to equation **1**, where K_{neu} and K_{ion} are the association constants for the neutral and ionised templates, respectively, and pK_a refers to the acid dissociation constant of the template in the absence of CD host.[29,31]

Equation 1

$$K_{app} = (1 - (1/(10^{pK_a - pH} + 1)))K_{neu} + (1/(10^{pK_a - pH} + 1))K_{ion}$$

Given that pK_a of **3** is 7.09,[29] 99.2% of **3** is in neutral form at pH 5 and 99.7% is deprotonated at pH 9.6. ^{1}H NMR spectroscopy titrations were, thus, performed for **3** with each CD at pH 5 and at pH 9.6 to determine values for K_{neu} and K_{ion} (Table 1 and Figs. S5–S10, ESI†). As expected from our experiments, and in line with reported values,[27–29] the affinity of α-CD for **3** was found to be highest a high pH. At low pH, **3** binds a little more strongly to β-CD than α-CD. Additionally, we observed very weak binding of **3** to γ-CD, which was strongest at high pH.

Using the tabulated values of K_{ion}, K_{neu}, and K_f values calculated from the equilibrium CD distribution in the absence of template (Section S4.1, ESI†), we applied our extended *DCLSim* approach to simulate DCLs at 7 different pH values

Table 1. Association constants, *K*, for cyclodextrins with **3** in D_2O at 25 °C [a]

Host	K_{neu} (M^{-1}) (*pH 5*)	K_{ion} (M^{-1}) (*pH 9.6*)
α-CD	165 ± 5	1625 ± 60
β-CD	265 ± 20	410 ± 50
γ-CD	40 ± 5	95 ± 20

[a] Based on ^1H NMR titrations carried out in acetate-phosphate buffer (50 mM).

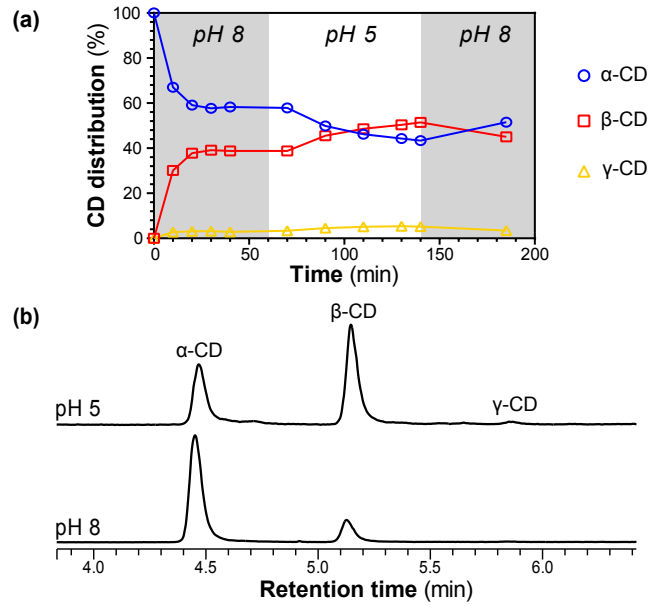

Figure 4 (a) Changes in the CD distribution in a DCL templated with **3** (2 mM) upon switching the pH of the reaction mixture. (b) Chromatograms (HPLC-ELS) showing the equilibrium distribution in DCLs templated with **3** (10 mM).

between pH 5 and pH 8 (Fig. 3b). Excellent agreement was found between the calculated distributions and the experimental results, accurately demonstrating the influence of pH on the DCL distribution (Fig. 3a,b). This calculation framework has the advantage that it may be used to predict the DCL distribution at different concentrations of starting material and/or template, and now also at different pH values.

To demonstrate the adaptive nature of this dynamic enzymatic CD system, a DCL (started from α-CD (2 mg/mL) templated with **3** (2 mM)) was initiated at pH 8, and after 60 minutes HCl (aq.) was added to adjust the pH to approximately pH 5, and after the next 80 minutes, the pH was adjusted back to pH 8 by addition of NaOH (aq.) (Fig. 4a). The system adapted readily to the new conditions, demonstrating the dynamic nature of the system. Finally, we report that an even larger change in the CD ratios can be achieved by using higher template concentrations. Equilibrium distributions for α/β/γ-CD of 39:53:8 at pH 5 and 68:29:3 at pH 8 were obtained in libraries with 10 mM of template **3** (Fig. 4b, and section S2.3, ESI†).

In conclusion, we have shown how pH can be used to predictably control the selective formation of different products in an enzyme-mediated DCL. The use of pH-responsive templates provides a novel, generalisable strategy to control the outcome of an enzymatic synthesis and a new approach to dynamically modulate α-CD/β-CD ratio in CD production.

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