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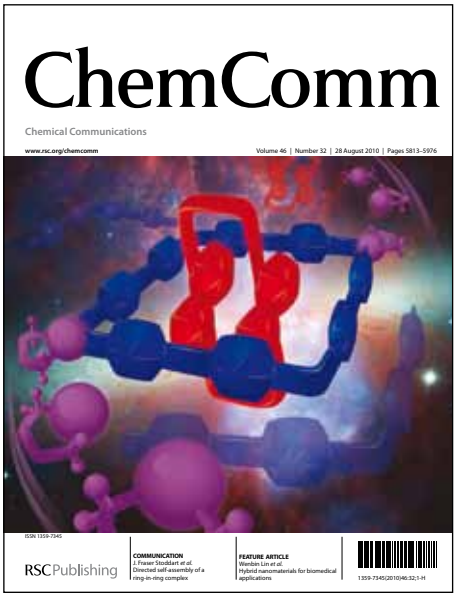

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COMMUNICATION

A fluorescence assay that detects long branches in the starch polysaccharide amylopectin

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Long α(1-4)-linked glucopyranose branches in the starch polysaccharide amylopectin can be detected by the specific binding of an anionic amphiphilic fluorescent probe. The probe forms spermidine-stabilised micelles in water resulting in fluorescence quenching. By extracting the probe from the micelles polysaccharides are detected in a "turn-on" fluorescence assay.

The selective molecular recognition and sensing of carbohydrates in aqueous solutions, including mono and disaccharides, oligosaccharides and polysaccharides, is extremely challenging due to their stereochemical diversity, extensive hydration, as well as their structural and conformational complexity.[1,2,3] Subtle structural variation in native polysaccharides, for example in the branched starch polysaccharide amylopectin, is linked to their physicochemical properties, their structural and functional roles in nature, their susceptibility to enzymatic degradation and their applicability for industrial purposes.[4]

We have previously described the selective recognition in water of long α(1-4)-linked glucopyranose branches in amylopectin using a small amphiphilic NMR probe, HPTS-$C_{16}H_{33}$, based on trisodium 8-hydroxypyrene-1,3,6-trisulfonate (Fig. 1a).[5] Sufficiently long α(1-4) linked glucans are capable of encapsulating hydrophobic guests by wrapping into single left-handed helices with the hydrophobic faces of the glucopyranose units oriented toward the centre of the helix to form a binding pocket reminiscent of a series of stacked cyclodextrins.[6] We reported that HPTS-$C_{16}H_{33}$ can bind in similar binding sites that can form in the dendrimer-like α(1-4)(1-6) branched starch polysaccharide amylopectin where there are sufficiently long stretches of consecutive α(1-4)-linked glucopyranose between the α(1-6) linked branch points or in sufficiently long non-reducing end (non-hemiacetal end) branches.[5] By quantifying the strength of the interaction, the number of binding sites, and the minimum number of consecutive α(1-4) glucosidic linkages required for a strong binding interaction it was possible to gain structural information on the investigated amylopectin molecule in terms the number of branches of defined length.[5a]

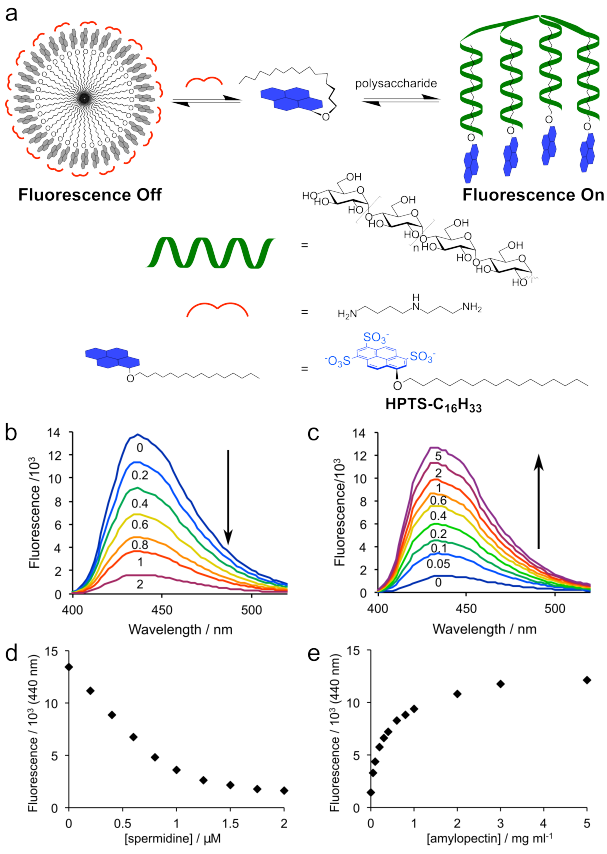

Fig. 1 (a) Spermidine binding to HPTS-$C_{16}H_{33}$ induces micelle formation and fluorescence quenching. Oligosaccharides that bind HPTS-$C_{16}H_{33}$ are detected by "turn-on" fluorescence as carbohydrate binding competes with micelle formation; (b) fluorescence quenching of a solution of HPTS-$C_{16}H_{33}$ (1 μM) in aqueous buffer (0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4) by addition of spermidine (concentrations labelled in μM); (c) fluorescence unquenching by addition of amylopectin (concentrations labelled in mg/ml as) to a spermidine (2 μM) quenched solution of HPTS-$C_{16}H_{33}$ (1 μM) in the same buffer; (d) binding isotherm for addition of spermidine; and (e) binding isotherm for subsequent addition of amylopectin.

Herein we describe how we have adapted this approach to polysaccharide structural analysis from an NMR-based method to a fluorescence-based assay. HPTS-$C_{16}H_{33}$ is strongly fluorescent in water at low concentrations with $\lambda_{max.\ em.}$ = 440 nm when excited at 350 nm.[7] However, polysaccharide binding of HPTS-$C_{16}H_{33}$ does not cause any fluorescence change. Köstereli and Severin recently reported that addition of spermine to dilute solutions of an analogous amphiphile, HPTS-$C_{20}H_{41}$, caused fluorescence quenching due to spermine binding-induced micelle formation.[8] It was suggested that spermine, a tetracation at neutral pH, binds at the surface of the micelles in a 1:2 (spermine:amphiphile) fashion, acting as an 'ionic glue' between the triply negatively charged HPTS moieties. We hypothesised that binding of a polysaccharide to HPTS-$C_{16}H_{33}$ could compete with polyamine-induced micelle formation resulting in a detectable "turn on" fluorescence effect (Fig. 1a).

We first examined the ability of different polyamines to induce fluorescence quenching of HPTS-$C_{16}H_{33}$. The tetracation spermine bound strongly to HPTS-$C_{16}H_{33}$ and a continuous variation (Job) plot confirmed a 1:2 binding mode as reported by Köstereli and Severin for HPTS-$C_{20}H_{41}$ (Fig. 2). The interaction with the trication spermidine was somewhat weaker and a Job plot analysis indicated a higher order binding ratio, possible 2:3 (Fig. 2b). These ratios correspond in each case to two protonated amines per amphiphile. Essentially no fluorescence quenching was observed upon exposure of HPTS-$C_{16}H_{33}$ to diamines 1,3-diaminopropane or 1,4-diaminobutane, which highlights the importance of a several cooperative binding interactions to induce the micelle formation and fluorescence quenching. For our competition-based assay we chose to use the weaker binding spermidine rather than spermine to induce fluorescence quenching, as this would lower the concentration of competing carbohydrate required to give a detectable fluorescence response. Addition of 2 equiv. of spermidine to a solution of HPTS-$C_{16}H_{33}$ (1 μM) in aqueous buffer (0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4) was found to cause a more than 90% decrease in the fluorescence emission at 440 nm (Fig 1a,c). In the presence of increasing amounts of amylopectin, the fluorescence emission was recovered (Fig. 1b,d), thus providing an optical means to detect the binding of the molecular probe.

While the presence of amylopectin clearly elicits a response in the fluorescence assay, we sought to confirm that this response resulted from the specific, length-dependent binding of HPTS-$C_{16}H_{33}$ to long linear α(1-4) linked branches in the polysaccharide. The fluorescence

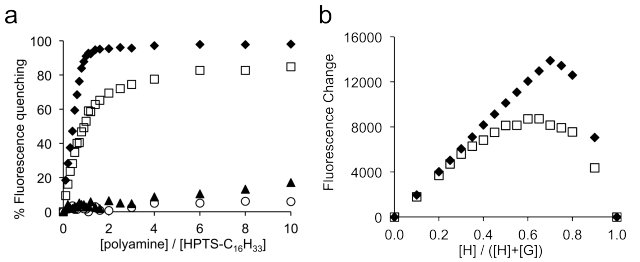

Fig. 2 (a) % Fluorescence quenching of aqueous buffered (0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4) solutions of HPTS-$C_{16}H_{33}$ (2 μM) (H) in the presence of polyamines (G): spermine (filled diamonds), spermidine (unfilled squares), 1,4-diaminobutane (filled triangles) and 1,3-diaminopropane (unfilled circles). (b) Continuous variation (Job) plots for the binding with spermine and spermidine. On the y-axis is plotted $F_{(no\ G)}\times[H]/([H]+[G])-F_{obs}$.

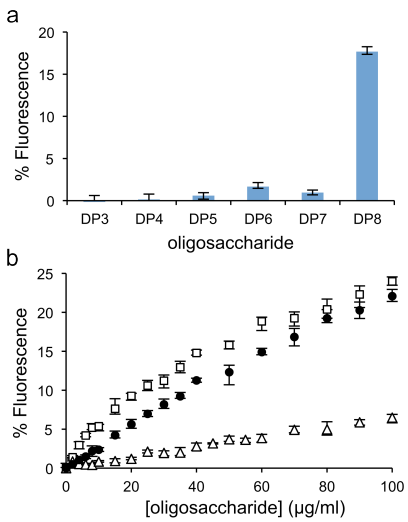

Fig. 3 (a) Relative % fluorescence emission of aqueous buffered (0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4) solutions of HPTS-$C_{16}H_{33}$ (1 μM) with spermidine (2 μM): (a) in the presence of linear maltooligosaccharides DP3 – DP8 (500 μM); (b) in the presence of increasing concentrations of maltooligosaccharide mixtures with average DP6 (unfilled triangles), mixture with average DP14 (unfilled squares) and α-cyclodextrin (filled circles). Values are normalised to 100% for 1 μM probe and no spermidine and to 0% for 2 μM spermidine and no saccharide. Average values from the analysis of three replicates are shown.

responses to the binding of short commercially available linear maltooligosaccharides, with degrees of polymerisation (DP) 3-8 were investigated. Fig. 3a shows the fluorescence emission at 440 nm of buffered aqueous solutions of HPTS-$C_{16}H_{33}$ (1 μM) and spermidine (2 μM) in the presence of each maltooligosaccharide (500 μM). It is possible using this assay to distinguish between maltooctaose and shorter maltooligosaccharides with DP3 – DP7 as a significant fluorescence recovery was only observed with maltooctaose under the conditions used. Binding studies conducted in D_2O by NMR spectroscopy showed a stronger interaction between the probe and maltooctaose (K_a = 640 M^{-1}), as compared with maltoheptaose (K_a ~ 220 M^{-1}) and maltohexaose (K_a ~ 70 M^{-1}) (Figs. S5 and S7-S9 in †ESI).

Maltooligosaccharides originating from starch degradation or synthesised by chemoenzymatic methods are produced as mixtures of different length oligosaccharides. Since these mixtures are very difficult to separate, and only commercial standards up to DP8 are readily available, maltooligosaccharides are frequently analysed as mixtures. Fig. 3b shows the possibility to distinguish between mixtures of oligosaccharides with different average lengths using our fluorescence assay. Two mixtures of maltooligosaccharides are examined: the first with a number average DP6 (ranging from glucose to DP22) and the second with a number average DP14 (ranging from maltotriose to DP32) (See Figs S1, S2 in †ESI for distribution analysis). The fluorescence response to the mixtures was compared with α-cyclodextrin, which binds HPTS-$C_{16}H_{33}$ with K_a = 2.8×10^4 M^{-1} as determined by NMR spectroscopy (Fig. S4 and S6 in †ESI). Clearly, the mixture of longer maltooligosaccharides gives a stronger fluorescence response. Since DP8 is the minimum oligosaccharide length that can bind HPTS-$C_{16}H_{33}$ and elicit a fluorescence response, only a weak fluorescence emisison would be expected for the average DP6 mixture, as a significant fraction of these oligosaccharides would be too short to interact with HPTS-$C_{16}H_{33}$. Meanwhile, the mixture with average DP14

gives a stronger fluorescence response and the curvature on the binding isotherm indicates that there is a population of longer maltooligosaccharides that can bind HPTS-$C_{16}H_{33}$ even more strongly than α–cyclodextrin.

In our previous work using NMR spectroscopy we found evidence that HPTS-$C_{16}H_{33}$ binding to amylopectin takes places specifically in hydrophobic helical binding sites formed in sufficiently long regions of consecutive α(1-4) linked glucopyranose, either between branch points or at the non-reducing ends of the branched polysaccharide. In developing a fluorescence-based assay it was important to establish a similar selectivity in the fluorescence response.

Samples of amylopectin were therefore systematically degraded with different hydrolytic enzymes that either hydrolyse or retain α(1-4) glucosidic linkages and the resulting products were analysed for the presence of long branches using our fluorescence-based approach (Fig. 4). α-Amylase (E.C. 3.2.1.1 from *Aspergillus oryzae*) is an *endo* α(1-4) glucopyranose hydrolase and cleaves internal α(1-4) linkages in amylopectin where several consecutive α(1-4) linkages occur.[9] β-Amylase (E. C. 3.2.1.2 from *barley*) is an *exo* α(1-4) glucopyranose hydrolase that sequentially cleaves maltosyl units from the non-reducing end of the polysaccharide until a branch point is reached. Pullulanase (E.C.3.2.1.41 from *Krebsiella planticola*) and isoamylase (E.C. 3.2.1.68 from *Pseudonomas sp.*) are both α(1-6) glucopyranose hydrolases that leave all α(1-4) glycosidic linkages intact and thus convert a branched polysaccharide to a mixture of linear fragments.

Samples of amylopectin (5 mg/ml) in D_2O were treated with each of the enzymes (while monitoring by NMR to confirm action of the enzymes, see Fig S3 in †ESI). Aliquots of the degraded samples were diluted and mixed with HPTS-$C_{16}H_{33}$ (1 μM) and spermidine (2 μM) in 0.5 mM pH 6.5 NaH_2PO_4/Na_2HPO_4 buffer to give 0.1 mg/ml solutions, then analysed for fluorescence emission at 440 nm. As seen in Fig. 4a, a fluorescence signal is only obtained in the presence of the native amylopectin and in the samples in which long α(1-4)-linked branches are cleaved from the polysaccharide but not further degraded (samples v, vi). Fig. 4b shows that fluorescence emission is furthermore visible by the naked eye upon irradiation at 365 nm, which suggests the potential of this approach for the monitoring of enzymatic starch degradation or to investigate the relative branching distribution in native starch samples.

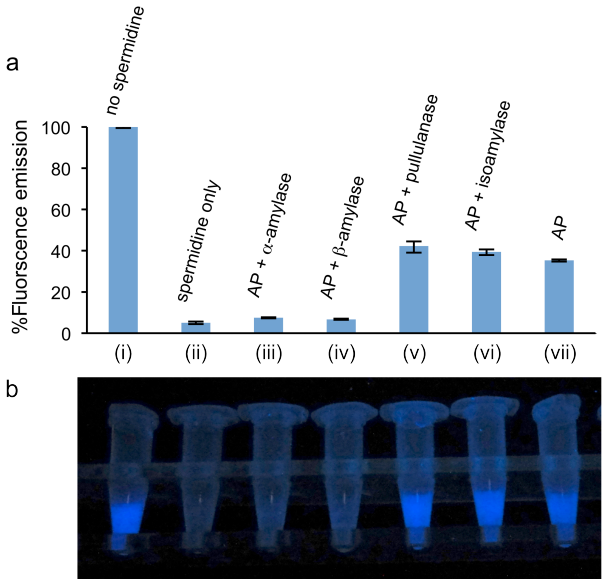

Fig. 4 (a) Fluorescence analysis of aqueous buffered (0.5 mM pH 6.5 mM NaH_2PO_4/Na_2HPO_4) solution of (i) HPTS-$C_{16}H_{33}$ (1 μM); (ii) with spermidine (2 μM) and with amylopectin (AP) (0.1 mg/ml) treated with different hydrolytic enzymes (iii) α-amylase, (iv) β-amylase, (v) isoamylase, (vi) pullulanase, (vii) no enzymatic degradation. Average values from analysis of three replicates are shown. (b) Photograph of the same solutions illuminated with UV light at 365 nm.

To conclude, we have developed a fluorescence-based approach to monitor for the presence of regions of consecutive α(1-4) linked glucopyranose (or long linear branches) in the starch polysaccharide amylopectin. The assay functions on the basis of a competition between two orthogonal molecular recognition events: spermidine-induced micelle formation and oligosaccharide binding. Fluorescence analysis enables detection at lower analyte concentrations, which permits the analysis of lower solubility poly/oligosaccharides, it uses only very small quantities of the amphiphilic probe per assay and allows for high throughput screening of multiple samples in parallel.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Experimental details, NMR spectra and titrations. See DOI: 10.1039/c000000x/

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