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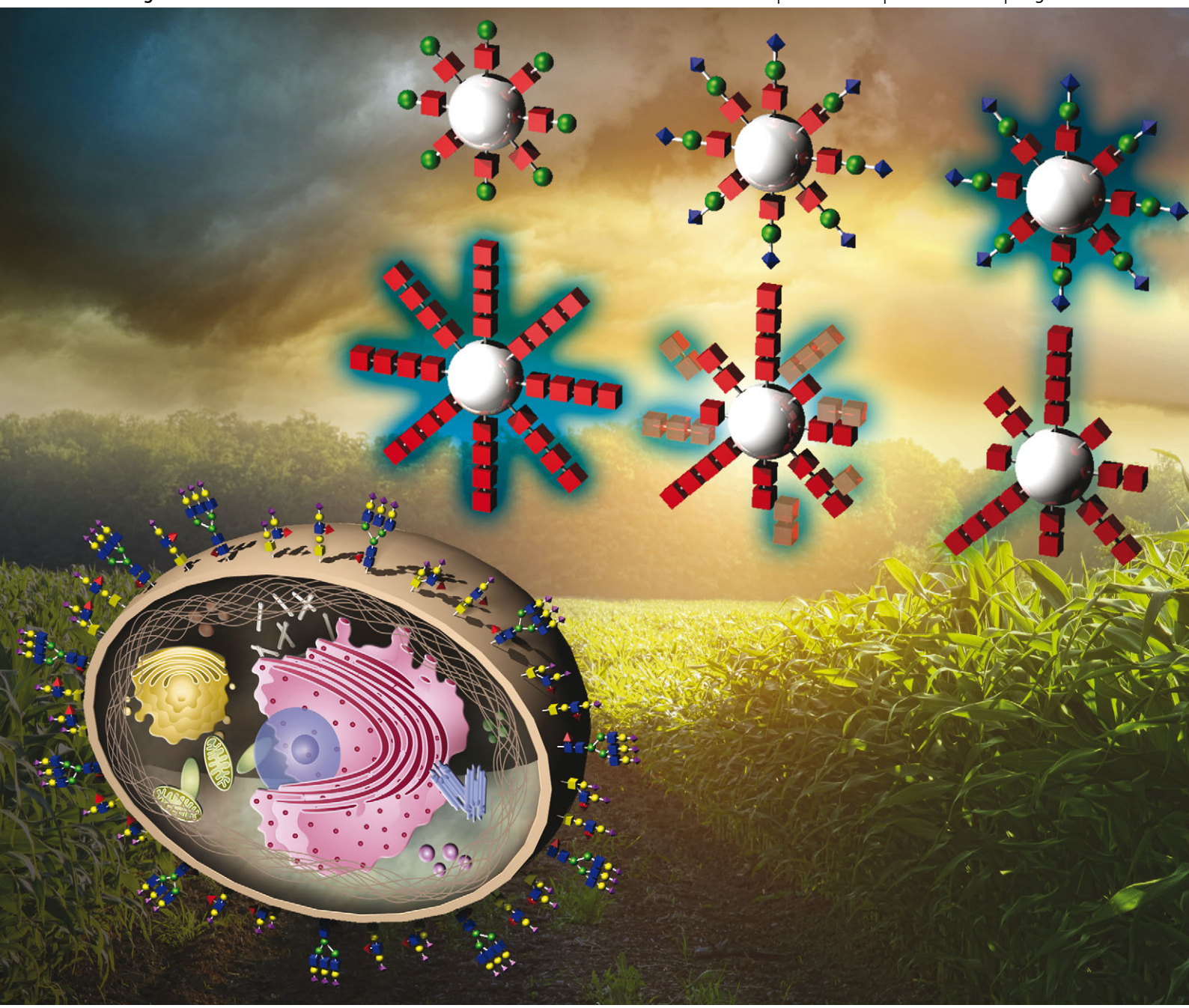

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A universal flow cytometry assay for screening carbohydrate-active enzymes using glycan microspheres†

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We describe a simple, multiplexed assay that integrates glycan synthesis, bioconjugation to microspheres, fluorescent chemical/biochemical detection and multiparameter flow cytometric analysis to screen activities of different families of carbohydrate-active enzymes.

Glycosyltransferases (GTs) and glycoside hydrolases (GHs) are carbohydrate-active enzymes responsible for the biosynthesis and breakdown of glycans in animals, plants and bacteria. By controlling the diversity of glycans, GTs and GHs regulate a number of cellular and disease processes in humans.[1] Given their diverse biological roles, several small molecule inhibitors of GTs and GHs are in clinical use for treatment of diseases such as tuberculosis, lysosomal storage diseases and influenza.[2,3] Further, these enzymes are being utilized for synthesis of novel carbohydrates for drug/vaccine development and diagnostics.[4,5] More recently, recombinant GTs and GHs have been gaining prominence in the biotechnology industry for their roles in the production of liquid biofuels and chemicals from plant-derived cellulosic biomass.[6,7] Manipulating cell-wall composition (by regulating GT activities) and increasing the efficiency of the enzymatic hydrolysis of cell-wall polysaccharides (by engineering improved GHs) are critical for lowering costs and improving production of biofuels.[7–9]

Current methods to screen GTs and GHs are plagued with limitations such as requirement for specialized equipment and training (in the case of mass spectrometry, HPLC, CE assays), special sample and waste handling (radiolabeled assays) and are currently available only for specific enzyme families (in the case of assays with colorimetric/fluorescent substrates).[3,7,9]

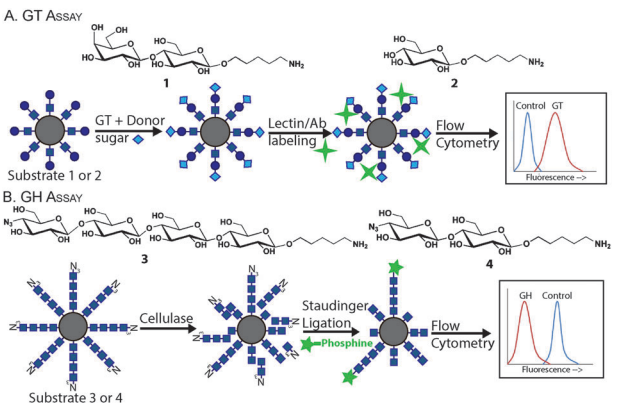

Fig. 1 Glycan microsphere-based flow cytometry assay. (A) Substrates for GT assay and schematic of representative GT assay using lactose-conjugated microspheres. (B) Substrates for GH assay and schematic of a representative GH assay using N_3-cellotetraose-conjugated microspheres.

To overcome these limitations and provide a universal screening platform for both GHs and GTs, we have developed a simple flow cytometry assay using glycan microspheres. Conventionally, microspheres have been extensively utilized for immunoassay development[10], and more recently, employed in applications such as genotyping[11] and enzyme assays.[12] In this study, we demonstrate for the first time the potential of an assay that combines covalent coupling of synthetic glycan substrates with microspheres with standard flow cytometry technologies to provide a general screening platform that can be easily adapted to any laboratory setting (Fig. 1 and Fig. S1, ESI†). Further, our approach permits easy multiplexing and is amenable to high throughput (HTP) screening of large enzyme libraries.

For GT assays, the glycan-functionalized microspheres are incubated with the GTs and an appropriate activated sugar nucleotide donor. GTs transfer the monosaccharide from the activated donor onto the acceptor sugar (coupled with microspheres) thereby generating a new glycan product on the microspheres. GT activity is tested using specific fluorescent lectins or antibodies that recognize the newly formed glycosidic linkages. The labeled microspheres are then analyzed by flow cytometry. The formation of the new

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glycosidic linkage leads to an increase in lectin binding indicated by an increase in fluorescence in the flow cytometry assay (Fig. 1a). Numerous lectins and antiglycan antibodies are currently available for characterizing a wide variety of plant and animal glycan products.[13,14] In addition, the assay can be adapted to screen GTs that accept unnatural activated monosaccharide donors containing chemical handles (*e.g.*, azides, biotin) that can subsequently be detected by labelled probes.[15,16]

For GH assays, we use glycans modified with a bio-orthogonal chemical handle, azide, on the non-reducing end as substrates. The azide-modified substrate coupled with the microspheres is detected by Staudinger ligation using a fluorescent phosphine probe.[17] In the presence of active GHs, the glycosidic bonds within the attached substrate are cleaved resulting in a loss of fluorescence due to loss of the azide from the microsphere surface, which is quantified by flow cytometry (Fig. 1b).

In this study, a panel of glycan substrates were synthesized for screening representative GTs and GHs (Fig. 1, compounds **1**, **2**, **3** and **4**). The reducing ends of all glycans are functionalized with a linker containing a terminal amine group (a C5–NH_2 linker). These amine-modified glycans are coupled with carboxyl polystyrene microspheres using standard carbodiimide-based peptide coupling protocols (see Fig. S6 and methods, ESI†).

The GT assay was validated by characterizing the activity of two recombinant GT enzymes: bacterial *Photobacterium damsela* α-2,6-sialyltransferase (6-ST) and human β-1,4-galactosyltransferase (GalT). The 6-ST enzyme transfers sialic acid (Neu5Ac) from the donor (CMP-sialic acid) to the amine modified-lactose substrate **1**, coupled with the microspheres, to form the product α-2,6-sialyl-lactose (Fig. S2 and S4A, ESI†). The newly formed Neu5Ac-α-2,6-Gal linkage is detected by adding fluorescein-SNA lectin. Flow cytometry analysis demonstrated a significant increase (~10-fold) in fluorescence of microspheres treated with 6-ST and CMP-sialic acid compared with the control (no enzyme) microspheres (Fig. 2a). Our assay is extremely sensitive and can detect as low as 25 fmol of 6-ST enzyme (Fig. S3, ESI†). The second enzyme tested, GalT, transfers galactose from the donor sugar (UDP-galactose) to the acceptor amine-modified glucose **2**, to form lactose (Fig. S2, ESI†). Detection using fluorescein-RCA-I lectin (specific to the newly formed Gal-β-1,4-Glc linkage) shows a significant increase in fluorescence compared to the control microspheres (Fig. 2b). Using 6-ST as an example, we further extend the assay to monitor GT reaction kinetics. We observe 6-ST activity within 5 minutes and saturation after ~30 minutes (Fig. 2c).

To validate the GH assay, we chose a cellulase–cellulose oligosaccharide system as a model GH enzyme–substrate system. We chemically synthesized substrate **3**, a bifunctionalized cellotetraose substrate with a C5-amine linker on the reducing end and an azide handle on the non-reducing end (Fig. S7 and S8, ESI†). Following conjugation of substrates (Fig. S4B, ESI†), we incubated the microspheres with four different purified recombinant *Clostridium thermocellum* cellulase enzymes to initiate the hydrolysis of substrate **3** attached to the microsphere. These cellulases release the products, azide–glucose, azide–cellobiose or azide–cellotriose, from the microsphere surface (Fig. S5, ESI†). Any remaining azides on the microsphere are fluorescently labeled by Staudinger ligation using Dylight 488-phosphine reagent and quantified using flow cytometry. As shown in Fig. 2d, incubation with all the four cellulases resulted in cleavage of substrate **3** and subsequent loss of fluorescence compared to microsphere samples not incubated

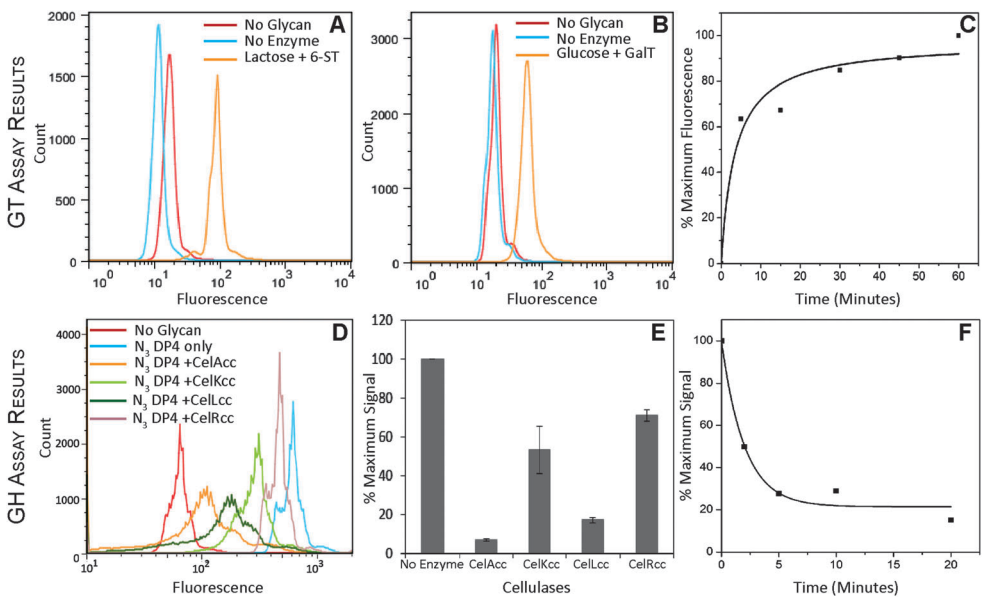

Fig. 2 Glycan microsphere-based GT and GH assay. (A) Analysis of GT activity of 6-ST using lactose-microspheres, (B) GalT activity using glucose-microspheres, (C) reaction time course for 6-ST activity. Data in 2A–C are representative of three separate experiments. (D) Flow cytometry analysis of GH activities of four recombinant cellulases using N_3-cellotetraose (N_3-DP4) conjugated microspheres: CelAcc_CBM3a; CelKcc; CelLcc_CBM3a; CelRcc_CBM3a. Data are representative of at least two separate experiments. (E) Comparison of GH activities of the four cellulases. Values are quantified by calculating the median of fluorescence values for approximately 40,000 events per sample and then normalizing the median values to that of the no enzyme control. Bars represent percentage with respect to control. Data show the average of two separate experiments ± SD. (F) Reaction time course for a representative enzyme, CelAcc_CBM3a. Data are representative of three separate experiments. See ESI† for detailed methods.

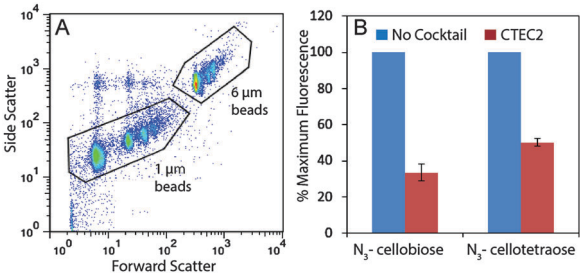

Fig. 3 Multiplexed enzymatic assay. (A) Dot plot (forward scatter *vs.* side scatter) depicts distinct regions for each microsphere population (1 µm *versus* 6 µm) based on microsphere size. (B) Multiplexed analysis of BG and EC activities of CTEC2. Values are quantified by calculating the median of fluorescence values for approximately 40,000 events per sample and then normalizing the median values to the value of the no CTEC2 control. Bars represent percentage with respect to control (100%). Data show the average of three separate experiments ± SD.

with any enzymes. Different enzymes showed different levels of loss of fluorescence indicating different hydrolytic activities for each enzyme with this substrate (Fig. 2e). For instance, while the cellulase CelAcc_CBM3a showed maximum activity, CelRcc_CBM3a showed minimal activity with substrate **3**. Using a representative cellulase, CelAcc_CBM3a, we demonstrate that our assay can be extended to study reaction time course of cellulase activity. We observe a significant loss of fluorescence in as little as 2 minutes after enzyme addition (~50% of initial value) with values dropping to 15–20% of the initial value by 20 minutes (Fig. 2f).

A key feature of our assay is the ability to multiplex, where multiple enzyme activities can be analyzed simultaneously. Multiplexing can be achieved using microspheres of different sizes or fluorescent properties where each individual microsphere population is coupled with a specific substrate. We demonstrate a proof-of-principle multiplexed assay by characterizing the cellulolytic activities of a cellulase cocktail (CTEC2) on two different substrates in a single experiment. CTEC2 contains a mixture of enzymes including beta-glucosidases (BGs) and exo/endocellulases (ECs). ECs do not work on cellobiose substrates and require longer cellulose oligomers as substrates while BGs typically hydrolyze cellobiose to glucose with decreasing affinities for longer cellulose oligomers. To profile the cellulolytic activities of CTEC2, we developed a multiplexed assay by combining microspheres of different sizes (1 µm and 6 µm). 1 µm microspheres were conjugated with a bifunctionalized cellobiose substrate, **4** (Fig. S7 and S9, ESI†) and the 6 µm microspheres were conjugated with substrate **3**. These two sizes were chosen because the individual microsphere populations cluster into two separate and distinct regions on a dot plot with minimal overlap between the clusters (Fig. 3a). Our assay shows that CTEC2 causes ~65% loss of fluorescence with the microspheres that contain **4** and ~45% loss of fluorescence signal with microspheres that contain **3** when compared with microspheres that were incubated only with buffer (no CTEC2 control) (Fig. 3b).

In conclusion, we demonstrate a simple, multiplexed, glycan microsphere-based bioassay for rapid screening of carbohydrate-active enzymes using standard flow cytometry technologies. Our assay is compatible with a number of biochemical (fluorescent lectins and antibodies) and chemical (*e.g.*, Staudinger ligation[17] or 'click-chemistry' Huisgen cycloaddition reactions[18,19]) methods for detecting the glycan products formed from GT/GH activities. Recent advances in HTP combinatorial glycan synthesis strategies[20] and in multiparameter, microsphere-based flow cytometry assays (*e.g.*, Luminex platform)[10] enable massive multiplexing of hundreds of discrete assays with a single sample. By integrating our assay with such technologies, one can envision a 'glycan library-on-a-bead' strategy with the ability to screen activities of large libraries of different GT and GH subfamilies using hundreds of unique glycan substrates. Such assays will be invaluable for advancements in GT and GH research for applications in drug and biomarker discovery, diagnostics and biofuels development.

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