

phosphate buffer (0.1 M) and Perfect Hyb hybridization buffer (Sigma, 1:1 v/v) for 5 h to give double-stranded DNA assembly on the surface. The resulting surfaces were rinsed with the hybridization buffer and immersed in a solution of hemin (1.2  $\mu\text{M}$ ) in buffer (25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO; pH 7.4) for 12 h at room temperature. The resulting system was further treated with doxorubicin (5, 5  $\mu\text{M}$ ) in phosphate buffer (0.1 M, pH 7.4) for 1 h at room temperature.

**Light-emission measurements:** Light emission was performed by using a photon-counting spectrometer (Edinburgh Instruments, FLS 920) equipped with a cooled photomultiplier detection system, connected to a computer (F900 v. 6.3 software). Before the samples analyses, the background light was recorded and integrated. This background was subtracted from the recorded integrated spectra of the respective samples. Sample analyses were performed by adding the DNAzyme solution (15  $\mu\text{L}$ ) or the modified surface to luminol (0.5 mM) and  $\text{H}_2\text{O}_2$  (30 mM) in buffer solution (3.3 mL; 25 mM HEPES, 20 mM KCl, and 200 mM NaCl; pH 9.0) in a cuvette.

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## Probing Protein–Carbohydrate Interactions with Microarrays of Synthetic Oligosaccharides

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Formerly a “neglected dimension” of biochemistry, recent years have seen growing interest in studying the biological function of carbohydrates and glycoconjugates. An emerging understanding of the physiological role of these biomolecules has uncovered their vital participation in a host of fundamental cellular processes. In the form of glycopeptides, glycolipids, glycosaminoglycans, and proteoglycans, glycoconjugates are known to be involved in inflammation,<sup>[1]</sup> cell–cell interactions,<sup>[2]</sup> signal transduction,<sup>[3]</sup> fertility, and development.<sup>[4, 5]</sup> Unfortunately, current methods for elucidating the biochemical roles of glycoconjugates are often cumbersome. This demonstrates the need to develop techniques that will satisfy this growing field of study by enabling rapid and facile exploration of biochemical events involving carbohydrates.

Inspired by the success of DNA and protein microarrays,<sup>[6, 7]</sup> the chip-based approach has been put forward as a useful tool in the emerging field of glycomics.<sup>[8–10]</sup> Nitrocellulose-coated slides have been employed for the noncovalent immobilization of microbial polysaccharides and neoglycolipid-modified oligosaccharides.<sup>[11, 12]</sup> Hydrophobic interactions have been utilized to anchor lipid-bearing carbohydrates on polystyrene microtiter plates.<sup>[13]</sup> Self-assembled monolayers presenting benzoquinone groups enabled the Diels–Alder-mediated immobilization of cyclopentadiene-derivatized monosaccharides on a gold surface.<sup>[14]</sup> Another covalent immobilization chemistry involved treating maleimide-functionalized mono- and disaccharide glycosylamines with a thiol-derivatized glass slide,<sup>[15]</sup> or, alternatively, thiol-functionalized carbohydrates with a self-assembled monolayer presenting maleimide groups.<sup>[16]</sup>

Our motivation for developing a system for arraying carbohydrates is based on the need to have microarrays that are fully

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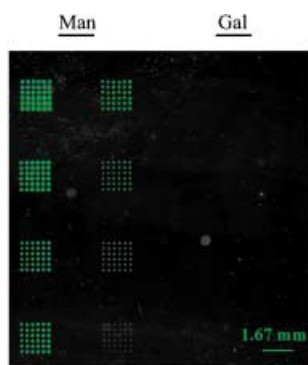
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compatible with existing high-throughput-screening (HTS) technologies and will enable the efficient immobilization of oligosaccharides drawn from solution-phase synthesis, automated solid-phase synthesis, and natural sources.<sup>[17]</sup> Such carbohydrate arrays will be useful tools in the identification of carbohydrate–protein interactions and will help to define the exact oligosaccharide structures involved in binding events. In addition, carbohydrate arrays could be used to rapidly screen for compounds that selectively inhibit protein–oligosaccharide interactions.

Here we describe a two-tiered approach in which a high-density microarray system is employed to study carbohydrate–protein interactions by using synthetic oligosaccharide structures immobilized covalently on bovine-serum-albumin (BSA)-coated glass slides by way of a hydrophilic thiol linker. These carbohydrate arrays are printed at high density by using DNA-microarray printing robotics and analyzed with conventional DNA-array scanners, thus by using technology available to many researchers. Specific interactions identified with the array are further analyzed by immobilizing the same carbohydrates to self-assembled monolayers and by using surface plasmon resonance (SPR) spectroscopy to characterize protein binding.

To demonstrate the ability of the system to detect protein–carbohydrate interactions, we used a well-known mannose/glucose-specific lectin, Concanavalin A (ConA). Microarrays were constructed through the maleimide derivatization of BSA-coated glass slides to create a thiol-reactive surface. Thiol-derivatized mannose and galactose (at varying concentrations) were printed as 120  $\mu\text{m}$  spots by using a microarray printing robot. Following incubation, the remaining maleimide groups were subsequently quenched with a solution of 3-mercaptopropionic acid to render the slides unreactive to cysteine-containing proteins. The carbohydrate microarrays were incubated with a solution of fluorescein isothiocyanate (FITC)-labeled ConA, thoroughly rinsed with buffer, dried by centrifugation, and scanned with a fluorescence slide scanner. As anticipated, FITC-labeled ConA was observed on the spots corresponding to immobilized mannose, while no fluorescence was associated with the spots that represented galactose (Figure 1). This result confirms that the microarray platform can be used for the immobilization of carbohydrates while maintaining specificity in carbohydrate–



**Figure 1.** Mannose and galactose, arrayed at concentrations ranging between 10 and 0.05 mM, on maleimide-activated BSA slides. Fluorescence after incubation with FITC-labeled ConA.

protein interactions. By utilizing proteins conjugated to fluorophores of nonoverlapping excitation and emission spectra, it is possible to extend this technology to include two or more colors, as previously demonstrated.<sup>[7]</sup> We observed very high signal-to-noise ratios in this experiment, presumably because of minimal nonspecific protein–surface interactions (Figure 1). Analogous observations have been made previously for protein microarrays that make use of derivatized BSA for the immobilization of small molecules.<sup>[7]</sup>

Having evaluated our carbohydrate microarrays with the above monosaccharide–lectin interaction, we sought to extend this system to include more biologically relevant and complex oligosaccharide structures. Here, we selected a series of high-mannose-type oligosaccharides, which are of key importance in the N-linked glycosylation pathway and also found on the glycoproteins of a variety of infectious agents such as the human immunodeficiency virus,<sup>[18]</sup> influenza virus, and trypanosomes.<sup>[19, 20]</sup> We chose the protein cyanovirin-N (CVN) as a model case for the study of protein–oligosaccharide binding events.<sup>[21]</sup> Isolated from the blue-green algae *Nostoc elliposporum*, CVN was found to bind the high-mannose oligosaccharides of the glycoprotein gp120, thereby inhibiting HIV's ability to infect target cells.<sup>[22]</sup> Natural and recombinant forms of CVN have been shown to irreversibly inactivate a wide variety of HIV strains while exhibiting minimal toxicity to host cells.<sup>[23]</sup> The ability of CVN to bind high-mannose oligosaccharides makes it an ideal test case for a carbohydrate array containing synthetic oligosaccharides of different lengths and complexity.

By using the aforementioned thiol-terminated linker, a series of high-mannose oligosaccharides (1–6) in varying concentrations were prepared. Arrays were printed on a BSA-coated slide and blocked with 3-mercaptopropionic acid. After incubation with 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diazas-indacene-3-undecanoic acid (BODIPY)-labeled CVN, fluorescence was detected at spots corresponding to the immobilized linear trimannoside **3**, hexamannoside **5**, and nonamannoside **6** (Figure 2), while the branched trimannoside **4**, mannose (**1**), and galactose (**2**) showed no binding activity.

Following the initial ligand identification, the same carbohydrates can be immobilized to the self-assembled monolayers for a more complete characterization of activity. Monolayers are well suited to quantitative investigation of protein–ligand interactions, because they allow excellent control over the density of immobilized carbohydrate and because they present the ligands in a homogenous environment at the interface. We adopted a previously reported method to immobilize the thiol-functionalized carbohydrates to a monolayer that presents maleimide and tri(ethylene glycol) groups in a ratio of 1:50.<sup>[16]</sup> MALDI-TOF mass spectrometry confirmed that the immobilization reactions proceeded in quantitative yield.<sup>[24]</sup> SPR spectroscopy was used to measure the binding of CVN to monolayers presenting the linear trimannoside **3**. A first experiment showed that the amount of bound CVN increases with the density of immobilized trimannoside **3** (Figure 3A). The ability to control the density of immobilized carbohydrate is an important advantage with the monolayer substrates, particularly because many proteins, including CVN, bind in divalent modes and show a strong



linker system that may be introduced in the solution-phase synthesis of any carbohydrate of interest, and can be extended to monolayer substrates for a more complete characterization of activity.<sup>[14]</sup> We envision that microarrays of diverse oligosaccharide structures will find a range of applications including epitope mapping of carbohydrate-specific antibodies<sup>[26]</sup> and ligand identification for cell-surface lectins obtained from cell lysates.

## Experimental Section

**Carbohydrate synthesis:** Thiol-terminated ethylene glycol-derivatized saccharides were prepared analogously to those described in the literature.<sup>[27]</sup> 2-(2-(2-Benzylsulfanyloxy)-ethoxy)-ethanol was substituted for 4-penten-1-ol. This substitution affords an ethylene glycol-modified thiol handle for covalent immobilization of the structures to a maleimide-modified surface.

**Functionalization of slides:** SuperAldehyde slides (TeleChem International) were immersed in phosphate buffered saline (1 × PBS, 50 mL) containing 1% BSA (*w/v*) and incubated overnight at room temperature. The slides were rinsed twice with distilled water (100 mL) and twice with 95% ethanol (50 mL), then dried under a stream of dry argon. Subsequently, the slides were immersed in anhydrous DMF (45 mL; Aldrich) containing succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (65 mg; Pierce Chemical) and *N,N*-diisopropylethylamine (100 mm; Aldrich). The slides were incubated in this solution for 24 h at room temperature, washed four times with 95% ethanol (50 mL), and stored in a vacuum desiccator until use.

**Microarray fabrication:** Thiol-functionalized carbohydrates were incubated for 1 h at room temperature with one equivalent of tris(carboxyethyl)phosphine hydrochloride (TCEP) in 1 × PBS, and printed on the maleimide-derivatized glass slides by using a MicroGrid TAS array printer. Prints were performed at 30% humidity by using either a 16- or 32-pin format, with a spot size of 120 and 300 μm spacing. Thereafter the slides were stored in a humid chamber at room temperature for 12 h, washed twice with distilled water, and then incubated for 1 h in 3-mercaptopropionic acid in 1 × PBS (1 mM, 50 mL) to quench all remaining maleimide groups. The slides were washed three times with distilled water (50 mL), twice with 95% ethanol (50 mL), and then stored in a vacuum desiccator until they were needed for the binding experiments.

**Detection of protein-carbohydrate interactions:** FITC-labeled ConA (25 μg mL<sup>-1</sup>; Sigma), was used in HEPES-BSA buffer (10 mM; pH 7.5; 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 100 mM NaCl, 1% BSA (*w/v*)) and BODIPY-labeled CVN (25 μg mL<sup>-1</sup>) was used in PBS (10 mM) containing BSA (1%, *w/v*). For all incubations, 0.5 mL of protein solution was applied to the slide by using a PC500 CoverWell incubation chamber (Grace BioLabs). Following a 1 h incubation at room temperature, the slides were washed three times with incubation buffer (50 mL), twice with distilled water (50 mL), and then centrifuged at 200g for 5 min to ensure complete dryness. The slides were scanned by using an ArrayWoRx fluorescence slide scanner (Applied Precision) to visualize fluorescence.

**SPR spectroscopy:** Monolayers presenting maleimide and tri(ethylene glycol) groups were prepared as described previously.<sup>[16]</sup> The thiol-terminated linear trimannoside **3** was immobilized to the monolayers by incubating the monolayers with Tris buffer (10 mM, pH 7.0) containing linear trimannoside **3** (1 mM) at 37 °C for 1 h. The monolayers were rinsed with water and dried under nitrogen flow before the SPR experiments. CVN-binding measurements were performed with a BIAcore 1000 instrument at 25 °C. PBS (10 mM,

pH 7.4) containing CVN at variable concentrations was passed over the immobilized linear trimannoside at a flow rate of 5 μL min<sup>-1</sup> for 20 min to allow association. The monolayer was then washed with 1 × PBS (pH 7.4) for 25 min to allow dissociation of CVN from the immobilized linear trimannoside **3**.

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