

**Encoded Fiber-Optic Microsphere Arrays for Probing Protein–Carbohydrate Interactions\*\***

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Carbohydrate-binding proteins and their glycoconjugate ligands play significant roles in a number of critical biological processes. Immune response, viral membrane fusion, glyco-protein homeostasis, and signaling all involve carbohydrate-binding protein mediation at key steps.<sup>[1]</sup> A more detailed understanding of the exact nature of carbohydrate–protein interactions is expected to render them attractive therapeutic targets. To facilitate these biochemical investigations, glyco-biologists require tools that will enable the simultaneous identification of carbohydrate-binding proteins and the oligosaccharide structures they bind.

Carbohydrate microarrays were introduced as a promising tool to aid in the discovery of oligosaccharide moieties necessary for carbohydrate-binding protein recognition.<sup>[2]</sup> Microarrays require small quantities of material, enable simultaneous screening of a defined set of structures against a host of potential binding partners, and, in some cases, are fully amenable to present high-throughput screening technologies, such as robotic printing and fluorescence scanning devices.

In the past year, several systems were described that present both natural and synthetically derived carbohydrate structures in array formats.<sup>[3]</sup> These systems differ in the mode of immobilization (covalent versus noncovalent), the surface upon which the immobilization was performed, and the

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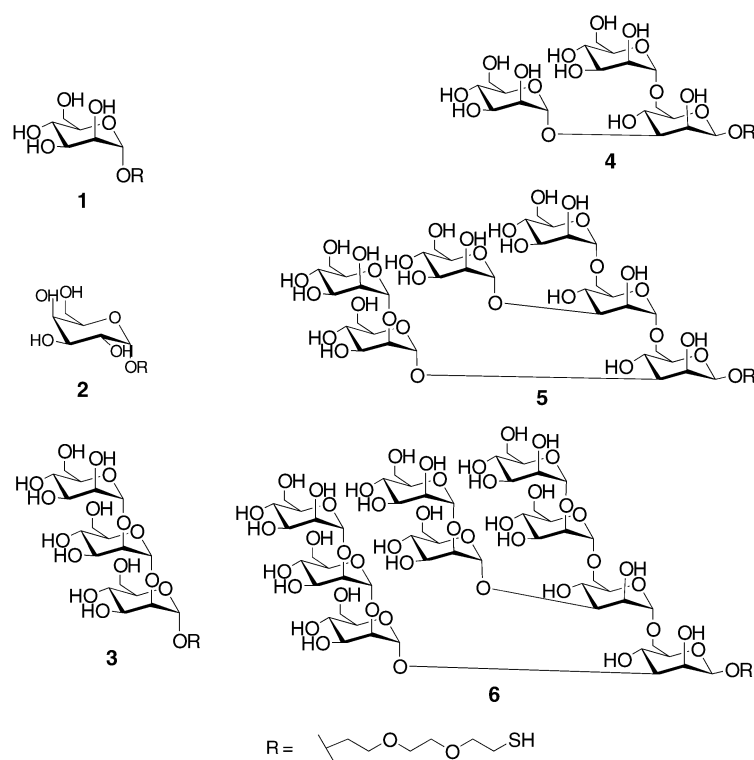
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means of detecting binding events (fluorescence and colorimetric detection). Herein we describe the use of fiber-optic microsphere arrays equipped with covalently immobilized synthetic oligosaccharides for the rapid analysis of protein–carbohydrate interactions.

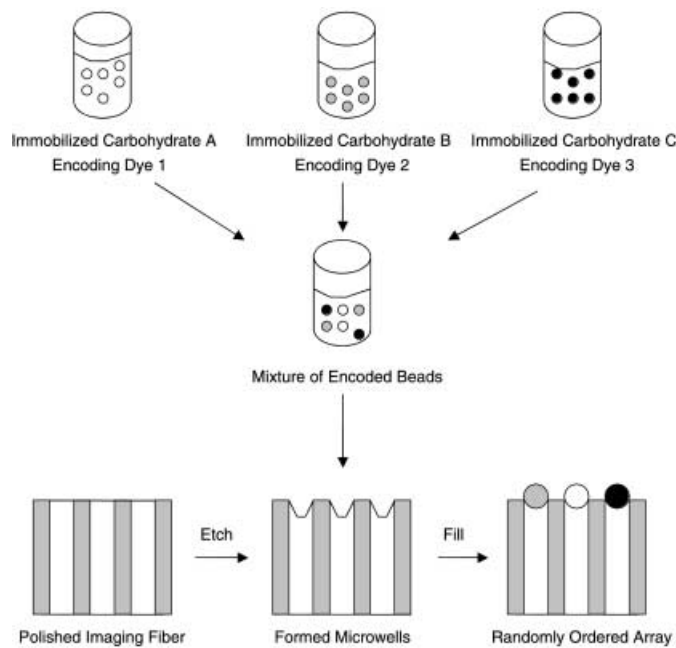
Fiber-optic random microsphere arrays have been described in detail for DNA hybridization detection.<sup>[4]</sup> Here we use arrays with different populations of 4.5- $\mu\text{m}$  microspheres of which each contains a unique carbohydrate structure covalently attached to its surface as well as an internally encoded spectral signature (an entrapped fluorescent dye with an emission maximum at 690 nm). The internal dye serves two purposes: it identifies the carbohydrate present on the surface of the bead surface and aids in determining the position of each type of microsphere in the array (Figure 1).

To demonstrate the utility of such arrays for studying protein–carbohydrate interactions, we examined two systems: the mannose-binding lectin concanavalin A (ConA), and cyanovirin N (CVN), a novel HIV-inactivating 11-kDa protein derived from the cyanobacterium *Nostoc ellipsosporum* with specificity for with a high mannose oligosaccharides.<sup>[5]</sup>

Mannose **1** and galactose **2** monosaccharides were prepared with a thiol-terminated ethylene-dioxy linker at the anomeric center (Scheme 1).<sup>[6]</sup> Each monosaccharide was coupled to commercially available maleimide-activated bovine serum albumin (BSA). The neoglycoproteins were then attached to encoded microspheres by using a water-soluble carbodiimide. This conjugation strategy was used to achieve adequate spacing between the underlying polymer support and the carbohydrates. The



**Scheme 1.** Carbohydrate structures used for the generation of randomly ordered microsphere arrays.



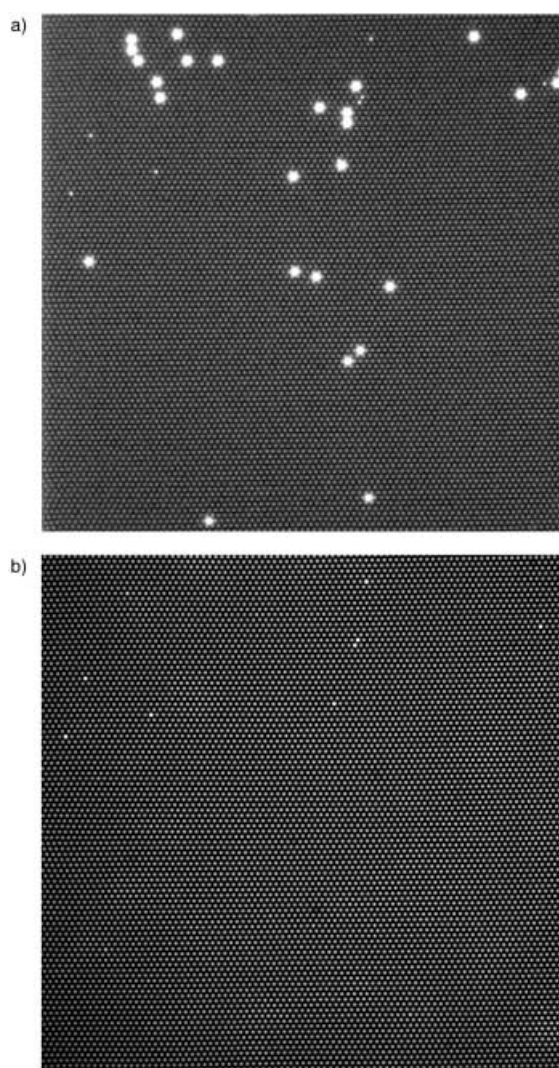
**Figure 1.** Schematic representation of randomly ordered microsphere arrays.

resulting carbohydrate-functionalized microspheres were used to form a randomly ordered fiber-optic microsphere array. ConA binding was detected by incubating the fiber-optic array in a solution of ConA labeled with Alexa488 at  $50\ \mu\text{g mL}^{-1}$  for five minutes. Then, the lectin solution was removed, replaced with a fresh buffer solution and the fluorescence signal at the Alexa 488 wavelength (520 nm) was measured. Only those beads bearing **1** were bound by labeled ConA (Figure 2). The intrinsic nonspecific-binding levels of fiber-optic arrays in this assay were very low and the specificity of carbohydrate–protein interactions was clearly observed as no ConA was detected in the empty wells or associated with beads functionalized with **2**.

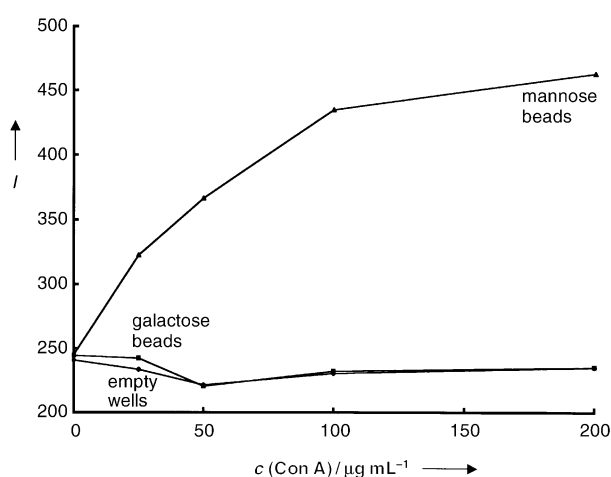
To determine the concentrations of labeled ConA required to observe signals easily discriminated over background, the fiber-optic arrays were incubated with a dilution series of labeled ConA ranging from 0 to  $400\ \mu\text{g mL}^{-1}$ . ConA concentrations as low as  $25\ \mu\text{g mL}^{-1}$  yielded fluorescence signals easily discernible over background (Figure 3). Even at very high lectin concentrations, no increase in fluorescence is observed associated with the beads presenting **2**.

After protein–carbohydrate interactions were detected with a simple lectin–monosaccharide system, five synthetic carbohydrates (**1,3–6**) were immobilized on microspheres carrying a unique internal code for each structure. Small aliquots of each of the five dispersions were mixed to form a random fiber-optic microsphere array and the array was probed with CVN.

CVN–oligosaccharide binding was assayed by incubating the array in a solution of BODIPY-labeled CVN at

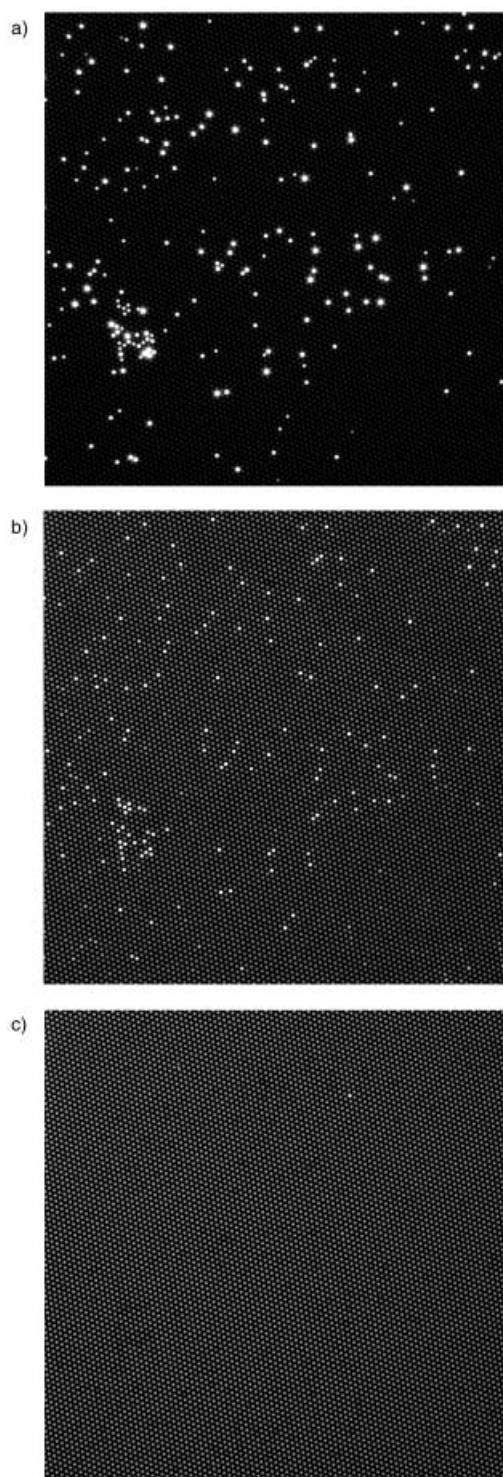


**Figure 2.** Internally encoded microsphere array presenting immobilized **1** and **2**. a) Fluorescence at 690 nm prior to ConA incubation; b) fluorescence at 520 nm, post-ConA incubation.

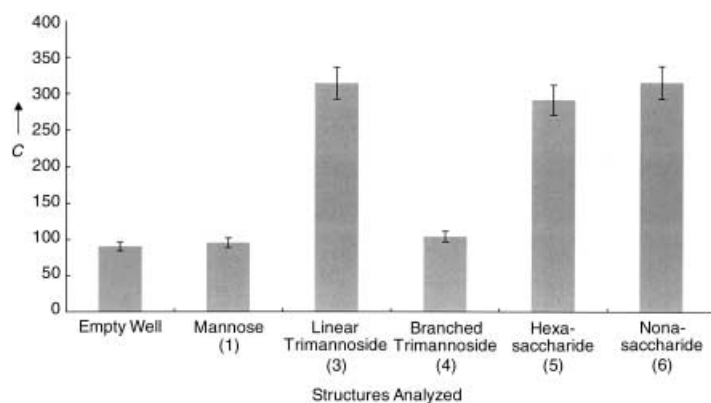


**Figure 3.** ConA dilution series. Microsphere array containing **1** and **2** was probed with ConA labeled with Alexa 488 at various concentrations (plotted fluorescence intensities normalized to background fluorescence).  $I$  = intensity of fluorescence (arbitrary units).

$50 \mu\text{g mL}^{-1}$  for five minutes before the CVN solution was removed and replaced with fresh buffer. Light at the BODIPY excitation wavelength (488 nm) was passed through the optical fiber bundle and the BODIPY emission was collected with a CCD camera. Three of the five structures (**3,5,6**) present were bound by CVN (Figures 4 and 5) in



**Figure 4.** Internally encoded microsphere array presenting **1** and **3–6** (see Scheme 1). a) Fluorescence at 690 nm prior to BODIPY-CVN incubation; b) fluorescence at 520 nm prior to CVN incubation; c) fluorescence at 520 nm post-CVN incubation.



**Figure 5.** Integrated fluorescence intensities from microsphere array. Fluorescence intensities were measured as detailed in the Experimental Section and normalized to background fluorescence. C = fluorescence counts, 520 nm.

accordance with isothermal microcalorimetry studies.<sup>[7]</sup> Beads that were not bound by CVN did not show any fluorescence signals over background levels (Figure 5).

In summary, we have demonstrated that randomly ordered fiber-optic microsphere arrays bearing immobilized synthetic oligosaccharides can be used to evaluate protein–carbohydrate interactions. The system described herein allows for the simultaneous evaluation of five distinct structures against a carbohydrate-binding protein with unambiguous results. While this study makes use of synthetic structures, future efforts towards functionalizing small quantities of complex oligosaccharides from natural sources (e.g., glycoproteins) should readily extend the applicability of the described platform for the study of protein–carbohydrate interactions.

## Experimental Section

**Carbohydrate synthesis:** Thiol-terminated ethyleneglycol-derivatized saccharides were prepared as described previously.<sup>[6]</sup> In the syntheses, 2-[2-(2-benzylsulfanyloxy)ethoxy]ethanol was substituted for pentenyl alcohol. This substitution affords an ethyleneglycol-modified thiol handle for covalent immobilization of the structures to a maleimide-modified surface or protein carrier.

**Neoglycoprotein preparation:** Maleimide-activated BSA and Tris(2-carboxyethyl)phosphane hydrochloride (TCEP) were purchased from Pierce Chemical. Compound **1** (50  $\mu\text{g}$ , 152 nmol) was incubated with 1 equiv TCEP in 10 mM HEPES buffer (pH 7.5) at room temperature for 1 h with constant mixing. This solution was added to 100  $\mu\text{g}$  maleimide-modified BSA in 100  $\mu\text{L}$  of the same buffer. The solution was incubated overnight at room temperature with constant mixing. Without further purification the encoded microspheres were added to this neoglycoprotein solution. This coupling chemistry was used for all structures in this study.

**Microsphere preparation:** Internally encoded QuantumPlex microspheres (4.4  $\mu\text{m}$ ) were purchased from Bangs Laboratories. Stock beads (100  $\mu\text{L}$ ) were washed by centrifugation/re-dispersion (3  $\times$  with 10 mM HEPES buffer, pH 7.5) and conjugated to neoglycoproteins with an excess of water-soluble carbodiimide. Beads were purified from excess neoglycoprotein by centrifugation/re-dispersion (3  $\times$  in HEPES buffer). Microspheres were stored in 10 mM HEPES buffer with 0.1% Tween 20 at 4  $^{\circ}\text{C}$  until use.

**Fiber-optic microsphere array formation:** Equal amounts (10  $\mu\text{L}$ ) of bead suspensions were mixed and 1  $\mu\text{L}$  of the mixture was added to

the etched end of an imaging fiber (about 24000 microwells, well diameter 5  $\mu\text{m}$ , Illumina, San Diego, USA). The mixture was left to dry before the excess beads were rinsed away with deionized water. About 30 beads of each type were present on the array.

**Detection of protein–carbohydrate interactions:** Images of the fiber-optic carbohydrate array were acquired with a custom-built epifluorescence microscope equipped with a xenon arc lamp and a CCD camera (Hamamatsu C4742-95-12-ER, Optical Analysis Corp., Nashua, NH). The system was controlled with IPLab software (Scanalytics, Fairfax, VA) which was also used for image processing. The positions of the microspheres were registered by exciting their internal encoding dye StarfireRed at 640 nm (emission maximum at 690 nm). The decoding of the different carbohydrate structures was achieved by analyzing the emission intensities at 690 nm. The array was incubated with the fluorescently labeled lectin at various concentrations for 5 min ( $V = 200 \mu\text{L}$ ). Then the lectin solutions were replaced with buffer and an image of the array was taken at the emission wavelength of the label (520 nm, exposure time 1 s). Fluorescence intensities in Figures 3 and 5 represent mean values of about 30 beads.

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