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Biotinylated Bi- and Tetra-antennary Glycoconjugates for *Escherichia coli* Detection

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Glycans cover the surface of all mammalian cells. Several toxins and pathogens use these glycans to bind and infect the cell. Using a versatile modular synthetic strategy, we have developed biotinylated bi- and tetraantennary glycoconjugates to capture and detect E. coli *and compared the capturing ability of these molecules to commercial polyclonal antibodies. Magnetic beads were coated with biotinylated glycoconjugate or antibody, and these beads were used to capture, isolate, and quantify bacterial recovery by using a luminescence assay. The glycoconjugate-coated magnetic beads outperformed antibody-coated magnetic beads in sensitivity and selectivity when compared under identical experimental conditions. Glycoconjugates could capture* Escherichia coli *from stagnant water, and the ability of a panel of glycoconjugates to capture a selection of pathogenic bacteria was also evaluated. To the best of our knowledge, this study represents the first comprehensive study that compares synthetic glycoconjugates and antibodies for* E. coli *detection. The glycoconjugates are also very stable and inexpensive. The results presented here are expected to lead to an increased interest in developing glycoconjugate-based high affinity reagents for diagnostics.*

Introduction

Early and accurate identification of bacteria is extremely important for environmental monitoring, food and water testing and point-of-care medical diagnostics.[1–3] Traditional bacterial culture technologies are the gold standard for detection, but it can take several days for the results to become available. The two main alternatives to cultures are immunoassays and real time PCR-based analysis.[4] Significant advances in micro/nanofabrication, instrumentation, and automation have rendered PCR-based analysis operable in real time.[4,5] However, PCR is often compromised by contaminants in real life samples and can require immunomagnetic separation and enrichment steps.[6] Antibodies are the traditional high-affinity reagents used to capture pathogens for PCR and ELISA. Polyclonal antibodies are purified from immunized animals, however, each production lot can vary, and separation of ultrapure biomaterials in large quantities can be expensive. Monoclonal antibodies do not vary from lot-to-lot, but might not be effective for pathogens capable of antigenic variation. In addition, antibodies exhibit poor shelf life and require refrigeration.[7] Refrigeration can be a major concern if immunoassays are to be used in remote areas, where stability without refrigeration is desired. Development of affinity reagents that are as selective as antibodies, robust, amenable to scale up, easily adaptable to existing biosensor platforms, retain their function in complex matrices, and are inexpensive could prove very beneficial for microbial detection.[8]

Several technologies are being intensively investigated to supplant antibody-based recognition elements. These include the development of stable, chemically modified antibody fragments, single chain antibodies that still exhibit high affinity,[9,10] affibodies,[11,12] molecular imprinted polymers (MIP),[13,14] aptamers,[15] protein receptors,[16,17] antimicrobial peptides,[18,19] and glycans. A notable and distinct advantage of glycans and peptide receptors is that these reagents function by mimicking the host receptor used by the pathogen to initiate the disease process. While some pathogens use protein receptors, many toxins and pathogens recognize and bind to cell-surface glycans, which decorate the surface of all mammalian cells.

Glycan-based receptor mimics have many advantages over antibodies. In direct contrast to antibody/aptamer/affibody/antibody-fragment based detection, in which the recognition epitope may or may not be related to the pathogenic potential of the microbe, binding to glycan receptors is required for virulence. Antigenic variation is unlikely to occur at the receptor binding sites, and receptor mimics could potentially distinguish pathogenic variants from nonpathogenic strains. Furthermore, glycan-based receptors are smaller than antibodies; this leads to greater surface coverage, facile scale up, and no lot-to-lot variation. Glycans also outmatch antibodies, or for that matter, any other naturally occurring biomolecule, in terms of information storage capacity. Indeed, nature uses glycans for a variety of communication processes, such as adhesion, communication, and differentiation.[20,21] Despite all these advan-

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tages, glycans in biosensing technologies have received scant attention in comparison to other recognition elements.

We have researched the use of glycans as potential recognition elements for various biosensors.[22,23] Recently, we reported that it is possible to develop synthetic glycoconjugates that exhibit antibody-like selectivity and sensitivity for toxins and viruses. We have demonstrated that synthetic glycoconjugates can detect and distinguish structurally homologous Shiga toxins (Stx1 and Stx2) from complex samples such as stool.[23] We have also demonstrated the ability of synthetic glycoconjugates to capture influenza virus.[24] In this study, we have synthesized bi- and tetra-antennary glycoconjugates and characterized their ability to capture larger entities (*E. coli*). In this context, it is important to note that capturing bacteria with glycans has been reported previously,[25–27] however, to the best of our knowledge, a direct head-to-head comparison of glycoconjugates and antibodies for *E. coli* detection has not been reported. It is essential to compare and contrast the features of novel affinity reagents to "gold standard" antibodies under identical assay conditions if the novel reagents are to be used as antibody substitutes. Additionally, we have screened a selection of bacteria against a panel of synthetic glycoconjugates and tested the ability of these glycoconjugates to detect bacteria from "real world" samples. This comprehensive study may prove to be useful to researchers interested in using glycans as integral components of biosensors.

Results and Discussion

Design and synthesis of biotinylated bi- and tetra-antennary glycoconjugates

A number of studies, including reports from our laboratories, have indicated that glycan-based recognition is highly dependent on three components: recognition, spatial presentation, and valency.[28,29] Each of these factors contributes to the overall binding event in a cooperative manner, and modulation of one or more factors can lead to discrete glycoconjugates that exhibit increased or decreased binding and specificity.[30,31] We have begun to create libraries of chemically defined glycoconjugates using a modular synthetic strategy that allows us to change one or more of the three critical elements without major modification of the synthetic strategy.

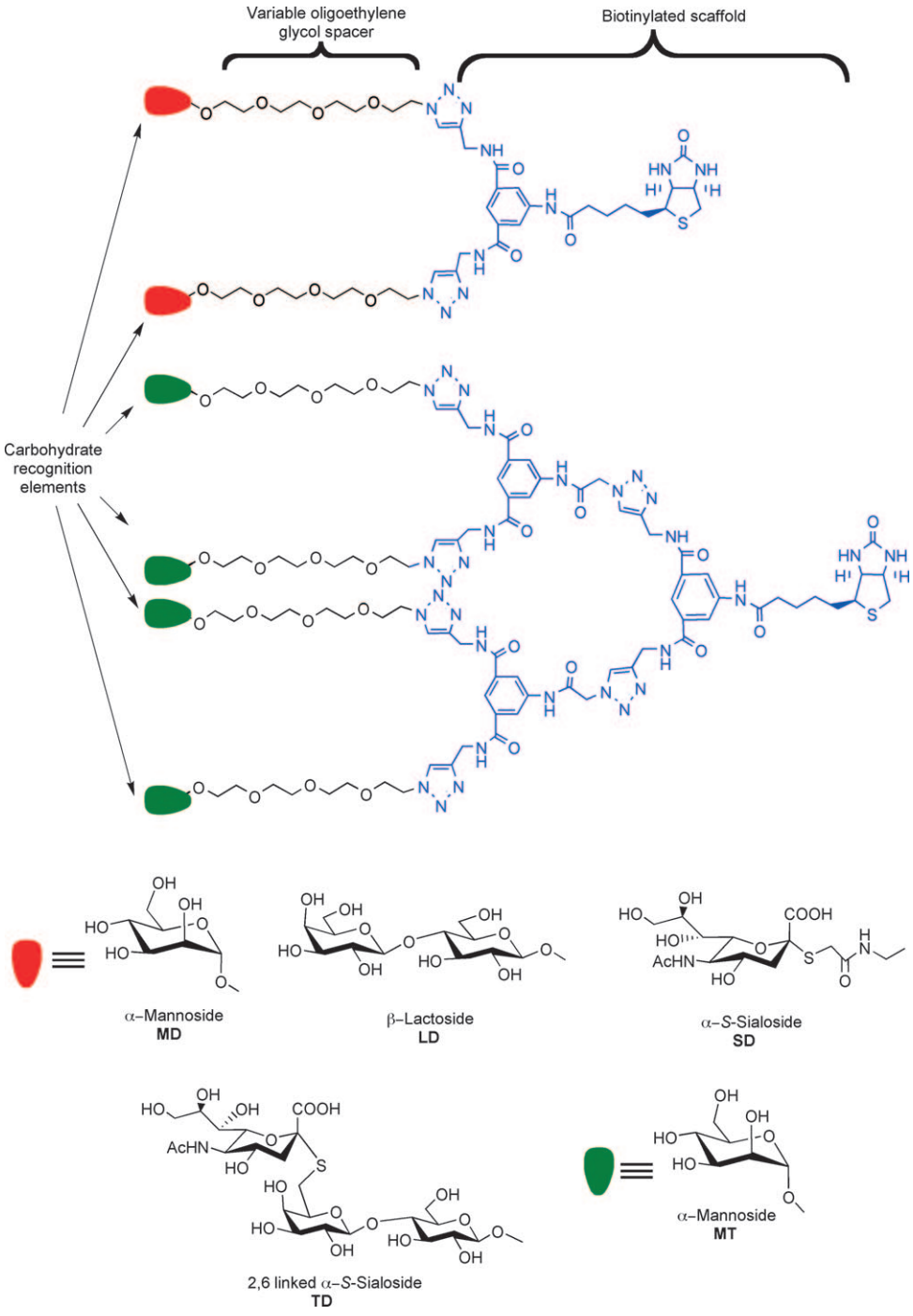
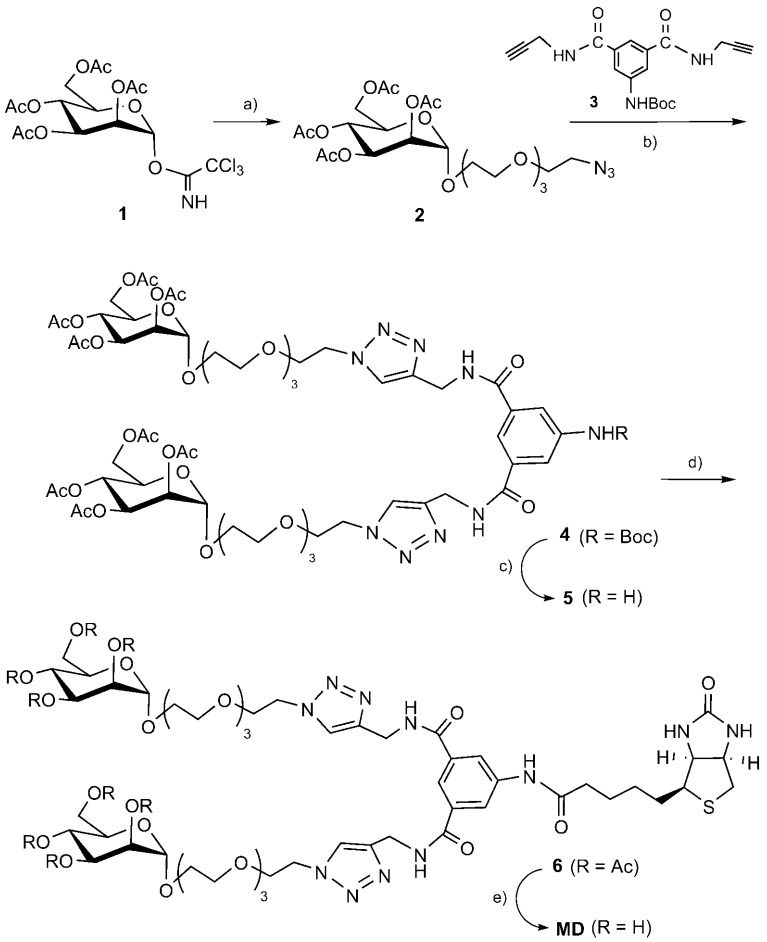


Figure 1. Representation of the bi- and tetra-antennary glycoconjugate structures. The red/green ellipse represents the glycan-recognition element, the biotinylated scaffold is in blue and the oligoethylene glycol spacer is in black.

The structures of the biotinylated bi- and tetra-antennary molecule used in this report are shown in Figure 1. The three modular components of these molecules are the glycan recognition element, linker, and biotinylated scaffold. The three glycans used in this study are mannose, lactose, and sialic acids. These basic components have been used to synthesize five chemically defined glycoconjugates that include the bi-antennary biotinylated α-mannoside (**MD**), β-lactoside (**LD**), α-thiosialioside (**SD**), a novel sialic acid trisaccharide (**TD**), and a tetra-antennary biotinylated α-mannoside (**MT**). We chose tetraoligoethylene glycol as a linker to reduce unspecific binding and to impart a degree of flexibility on the recognition molecules for a better fit in the binding sites. The spacer element also connects and separates the biotin from the recognition motifs. Finally, a biotinylated scaffold forms the third component of the designer molecule. We have used a benzene-like scaffold as a starting point to render some rigidity to the scaffold. Specifically, we chose 5-amino-isophthalic acid because the two acid functionalities are in the *meta* position, which reduces the possible formation of a lactone byproduct. The dimeric scaffold core is very versatile, as it can be extended to a tetrameric scaffold easily; indeed, we have synthesized a tetra-antennary α-mannoside (**MT**) in addition to **MD**.

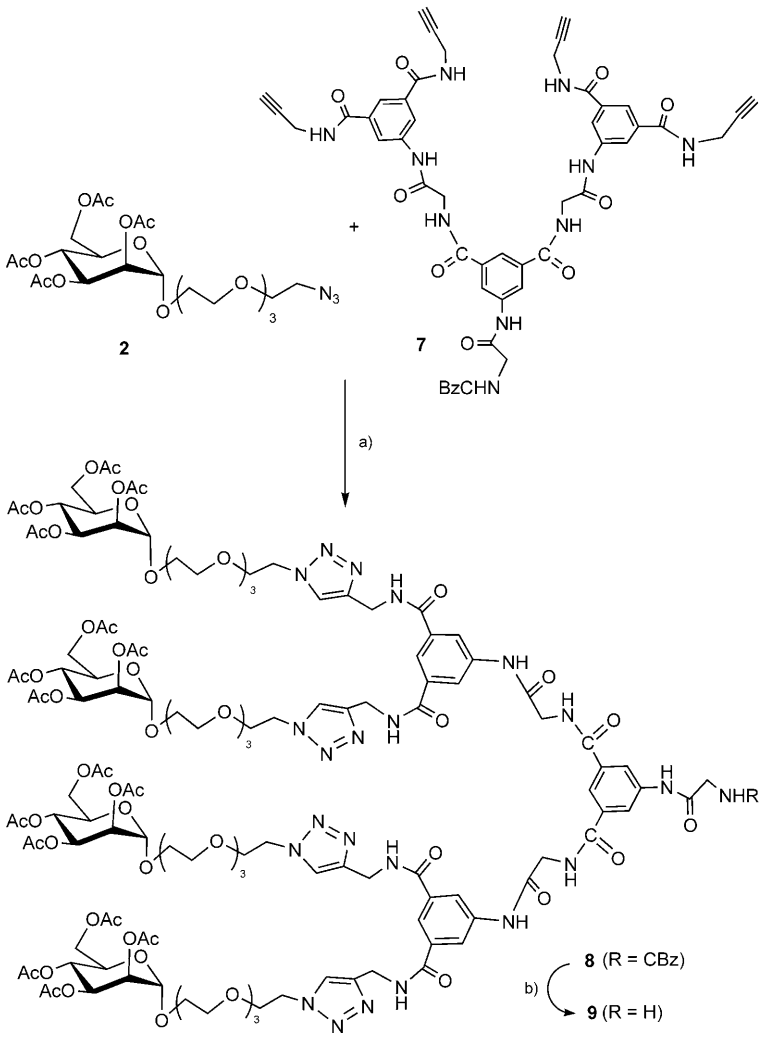


Scheme 1. Synthesis of the biotinylated bi-antennary α-mannoside, **MD**. Reagents and conditions: a) $H(OCH_2CH_2)_4N_3$, TMSOTf, DCM, 0 °C, 1.5 h, 65%; b) $CuSO_4$, sodium ascorbate, THF:H_2O, 48 h, 77%; c) TIPS, TFA, DCM, 0 °C→RT, 48 h, 69%; d) CDMT, NMM, D-biotin, THF:DMF, 0 °C→RT, 48 h, 72%; e) NaOMe, MeOH, RT, 24 h, 77%.

An additional key feature we desired was a simple and effective bioconjugation without the need for optimization. Traditional coupling methods to biosensor surfaces include amine, thiol, or the more recent click coupling. While these bioconjugation methods have been used extensively, they require optimization of reaction conditions to achieve homogeneity, appropriate orientation, and density. Experimental variation could prove especially problematic in studying and understanding glycan–pathogen interactions, as these interactions are highly dependent on the density and spatial orientation of the sugar residues. Therefore, we preferred attaching biotin to these molecules, so that multiple sensor platforms could be compared without having to resort to complex bioconjugation techniques or optimization. In addition to minimal manipulation, the avidin–biotin system is well-studied and characterized.[32] Avidin affords multivalency, which is an essential factor for studying glycan–protein interactions; a single avidin molecule binds four biotin molecules. Third, avidin-coated magnetic beads, fluorescent nanoparticles, and microwell plates are commercially available for high-throughput screening of analytes that bind to biotinylated compounds.

The synthesis of the bi-antennary α-mannoside **MD** is shown in Scheme 1. Briefly, treatment of known mannose trichloroacetimidate[33] **1** with the aglycon in the presence of TMSOTf as promoter resulted in the α-mannoside **2**, with 100% stereoselectivity. The anomeric proton resonated at 4.8 ppm ($J_{1,2}$ = 3.6 Hz) and 97.7 ppm in the ^1H and ^{13}C NMR spectra, respectively, which confirmed the existence of the α-mannoside. Coupling of the dimeric scaffold bearing two alkynes, **3**, with two equivalents of the azide terminated mannoside **2** resulted in **4**. A simple sequence of steps was performed to attach biotin to the bi-antennary complex. First, removal of the protecting *tert*-butyloxy group was performed by using standard conditions to yield the free amine **5**, which was coupled with D-biotin to yield the completely protected biotinylated derivative **6**. Deprotection by using Zemplén conditions gave **MD** in excellent yield (HRMS for $[C_{63}H_{91}N_9O_{30}+H]^+$ = 1244.5726). The final product was obtained in overall yield of 13.4% over seven steps starting from mannose pentacetate. A similar strategy was used to synthesize the other bi-antennary glycoconjugates (Figure 1).

Since we were interested in increasing capturing efficiency, we also synthesized a tetrameric complex. The glycoconjugate **MT** has four mannosides (Figure 1), which is twice the number of recognition elements than **MD**. This number increases dramatically when magnetic beads that bear 2×10^6 streptavidin molecules are used, as in our system. Our initial strategy to synthesize a biotinylated tetrameric ligand is shown in



Scheme 2. Attempted synthesis of the biotinylated tetra-antennary α-mannoside. Reagents and conditions: a) $CuSO_4$, sodium ascorbate, THF:H_2O, 48 h, 54%; b) H_2/Pd/C, EtOH, EtOAc, quantitative.

Scheme 2. Here, we decided to synthesize the tetrameric scaffold first; this was followed by "clicking" the azide with the α-mannosides and attachment of biotin in the last step.

To this end, the previously reported tetrameric scaffold **7** was utilized. This scaffold has four alkynes to couple to four azide-bearing molecules. We "clicked" the azide-bearing mannose ligand **2** to the tetrameric scaffold; this resulted in the tetravalent glycoconjugate **8** in appreciable yields. In line with the proposed strategy, we removed the carbobenzyloxy (Cbz) protecting group of **8** to obtain the free amine **9**, and coupled the D-biotin using standard peptide synthesis. Unfortunately, several attempts to couple **9** with D-biotin by using various reaction conditions resulted in negligible amounts of the desired biotinylated product. We attribute these failures to steric hindrance. Presumably, the free amine of the dendron is not readily accessible for coupling. Therefore, we modified our strategy, as shown in Scheme 3, and synthesized a biotinylated scaffold with two alkynes first and subsequently attached the azide-bearing recognition elements to the biotinylated scaffold. To this end, the Boc protecting group of **3** was removed, and the free amine of the resulting compound (**10**) was coupled with D-biotin to yield a biotinylated scaffold (**11**) with two alkyne functionalities. Next, the free amine of the divalent mannoside derivative **5** was extended to an azide through reaction with bromoacetylbromide and conversion of the resulting bromide (**12**) to the azide **13** in reasonable yields. The bi-antennary mannoside derivative (**13**) was then ready for treatment with any alkyne-bearing compound. Thus, treatment of 2.2 equiv of **13** with the divalent scaffold **11** resulted in **14**. Global deprotection by using Zemplén conditions resulted in the desired tetravalent compound, **MT**. The successful synthesis of the biotinylated tetravalent glycoconjugate demonstrates the versatility of this modular strategy.

Capture assays

Enrichment steps to capture microbes from samples often employ antibody-coated magnetic beads (Figure 2). We examined the ability of glycoconjugates to replace antibodies in the magnetic separation and enrichment step. Micrometer-sized commercial streptavidin-coated magnetic beads were incubated with the biotinylated glycoconjugates to yield "glycomagnetic" beads that were completely covered with glycans. The coated beads were isolated with a standard magnet, washed, and incubated for 10 min with two isogenic strains of *E. coli*, ORN178 and ORN208.[34] The ORN178 *E. coli* bear numerous pili, which possess the terminal FimH fimbrial adhesin receptor. This receptor binds preferentially to α-mannosides and not to β isomers.[35–38] Strain ORN208 is mutant for pilus expression.

In initial experiments, we observed that strain ORN178 mediated the aggregation of beads coated with mannose-bearing compound **MD** within minutes of addition to the beads (Figure 3A) while strain ORN208 did not (Figure 3B). Bacterial aggregation has been shown to be dependent on multivalency, and these results suggest that a single bacterium can bind to multiple beads. Aggregation was further examined by using environmental scanning electron microscopy (ESEM). As seen in Figure 4, at low magnification **MD**-coated beads were aggregated by the pilus-expressing strain ORN178 (Figure 4A) but not the pilus mutant ORN208 (Figure 4B). At high magnification individual bacteria

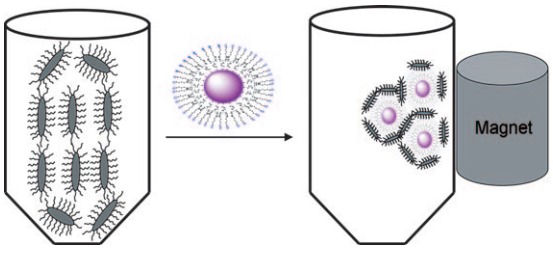
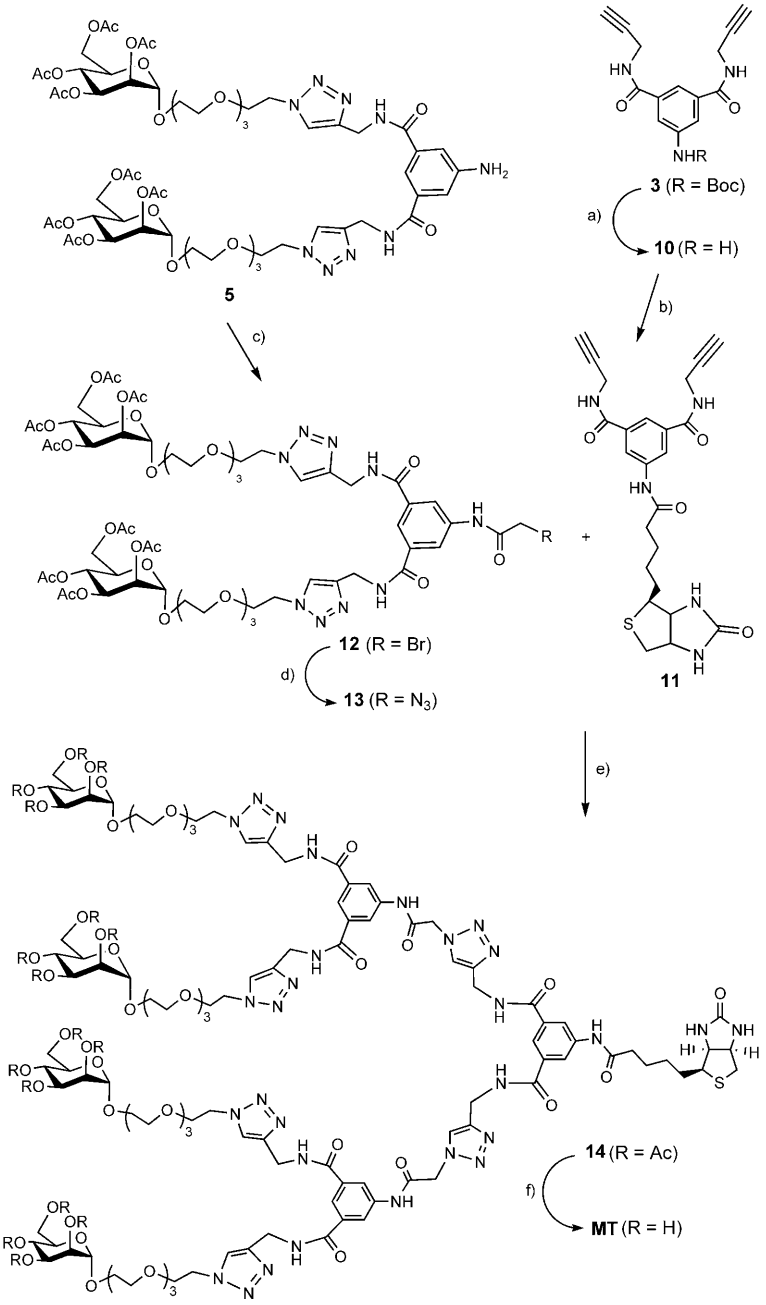


Figure 2. Cartoon representation of the capture of *E. coli* by using glycoconjugated magnetic beads and a magnet to isolate the "magnetized" *E. coli*.



Scheme 3. Synthesis of the biotinylated tetra-antennary α-mannoside, **MT**. Reagents and Conditions: a) TIPS, TFA, DCM, 0 °C→RT, 65%; b) D-biotin, CDMT,NMM, THF:DMF, 0 °C→RT, 70%; c) Na_2CO_3, CH_3CN, 0 °C→RT; d) NaN_3, DMF, 0 °C→RT, 32% over two steps; e) $CuSO_4$, sodium ascorbate, THF:H_2O, 48 h, 57%; f) NaOMe, MeOH, RT, 37%.

can be seen trapped between the glycomagnetic beads (Figure 4C). The aggregation experiments clearly demonstrate that these glycomagnetic beads can be used for capture and isolation of pathogens from other complex matrices.

Comparison of antibody- and synthetic glycoconjugate-coated magnetic beads as capture reagents

We compared the efficiency of "glycomagnetic" beads to standard antibody-coated beads for the capture of bacteria. Magnetic beads were coated with biotinylated biantennary mannose glycoconjugate (**MD**) or commercial biotinylated antibody (**Ab**) and incubated with known concentrations of bacteria. Next, the captured, aggregated bacteria were isolated by using a bar magnet, and washed with buffer. We used the BacTiter-Glo® assay to quantify bacterial recovery.[39] In this assay, the presence of ATP is used as an indicator of metabolically active bacterial cells. The enzyme luciferase oxidizes luciferin, which in turn produces light in a reaction that is dependent on ATP. The amount of light produced is proportional to the amount of ATP present and can be quantified by using a luminometer. Therefore, an advantage of this assay over other fluorescent-based assays is that viable bacteria can be distinguished from dead bacteria. Also, the assay uses minimal reagents, is user friendly and rapid; unknown samples can be processed within 20 min—a critical requirement for rapid diagnostic kits. The sensitivity of bacterial recovery with biotinylated antibody (**Ab**) or biotinylated bi-antennary mannose glycoconjugate (**MD**) coated on streptavidin magnetic beads was determined under identical experimental conditions. As shown in Figure 5A, the limit of detection with the Bactiter-Glo reagent was 10^5 CFU mL^{-1} for both **Ab** and **MD**. However, the glycoconjugated beads captured significantly more bacteria at all concentrations, especially at higher *E. coli* concentrations. At 10^7 CFU mL^{-1}, the sensitivity of the glycoconjugated beads (**MD**) was twice the sensitivity of the antibody conjugated beads (**Ab**). The antibody was also able to capture the nonpiliated mutant, ORN208 (Figure 5B), and although capture of the mutant appeared to be less efficient than capture of the piliated strain ORN178, the differences were not statistically significant.

We determined the capture efficiency by comparing the luciferase activity of the bacteria captured on beads to the activity of a known amount of bacteria pipetted directly into the microtiter wells. When all three trials were averaged, the **Ab**

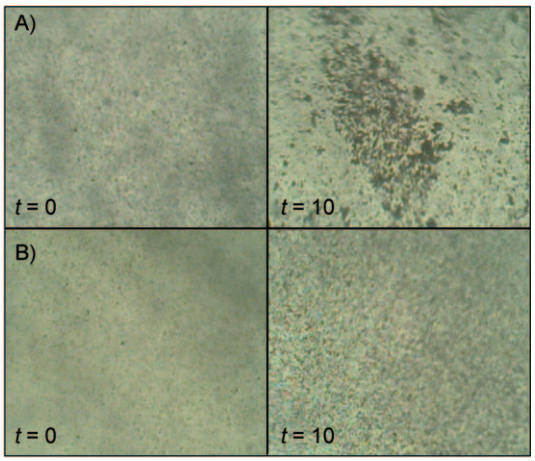


Figure 3. Agglutination of the piliated *E. coli* strain (ORN178-A) versus the nonpiliated (ORN208-B). Both strains were incubated with streptavidin-coated magnetic beads attached to biotinylated bi-antennary mannose-conjugated magnetic beads (**MD**). The concentration of *E. coli* was 10^7 CFU per well. Pictures were taken at 0 and 10 min with a premiere digital 10X microscope eyepiece inserted into a Nikon TMS microscope. Agglutination was observed with the piliated strain.

was found to capture 15.2% of the bacteria when added at 10^7 CFU mL^{-1}, 8.2% when added at 10^6 CFU mL^{-1}, and 5.3% when added at 10^5 CFU mL^{-1}. The efficiency of capture was greater for **MD**: 33.7% of the bacteria were captured when added at 10^7 CFU mL^{-1}, 18.9% when added at 10^6 CFU mL^{-1}, and 25.7% when added at 10^5 CFU mL^{-1}. Similar recoveries were observed with antibodies from a different commercial source (data not shown) and the increased capture by the glycoconjugated beads is likely due to the smaller size of the glycoconjugate, which results in a higher packing density compared to the larger antibody molecules.

We were also interested in improving the sensitivity of detection. Initial studies used nondividing, stationary-phase bacteria. Since the luciferase assay measures metabolic activity, we incubated the captured bacteria with LB media containing glucose (20%) for 1 h at 37 °C prior to performing the BacTiter-Glo® assay and compared these cells to cells incubated with PBS buffer. The results are shown in Figure 6. Clearly, sensitivity can be increased by incubating the bacteria in media. Greater sensitivity can be achieved by plating the bacteria and performing colony counts, however, this requires overnight incubation (data not shown).

Comparison of bi- and tetra-antennary glycoconjugate-coated magnetic beads as capture reagents

We were interested in understanding if the increase in the number of glycans on the magnetic beads would lead to an increase in capturing ability. Therefore, we used **MT**, which has twice as many recognition elements as **MD**. Results of the capture study are shown in Figure 7. At lower concentrations of *E. coli*, there was no discernable increase in capture efficiency, however, at higher concentrations, increased capture was apparent with **MT**. These data suggest that **MT** has a greater capture capacity than **MD**, but both molecules are equally effective when lower concentrations of bacteria are used. We are currently trying to increase the capture efficiency by using magnetic nanoparticles, as they have larger surface areas[40,41] and could presumably increase the capture efficiency.

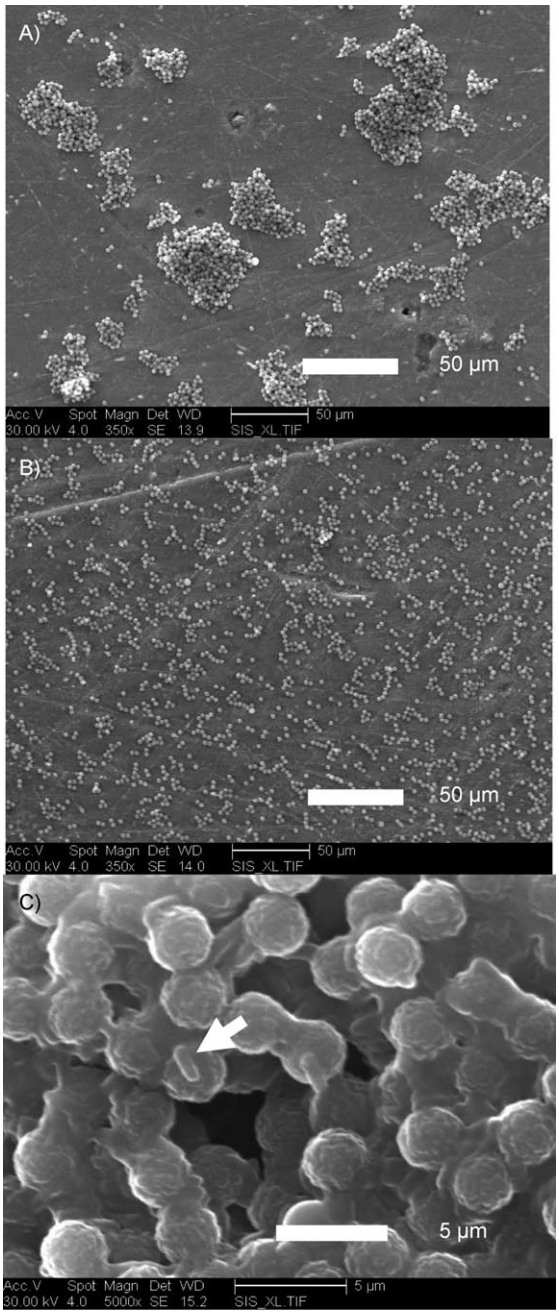


Figure 4. ESEM pictures obtained from biotinylated bi-antennary mannose-conjugated magnetic beads (**MD**) exposed to bacteria: A) 10^7 piliated *E. coli* ORN178, or B) mutant ORN208 were incubated with biotinylated bi-antennary mannose-conjugated magnetic beads (**MD**, 25 µL) and rinsed 2X with H_2O to remove salt; C) ORN178 were incubated with biotinylated bi-antennary mannose-conjugated magnetic beads (**MD**) and rinsed 2X in PBS to preserve bacterial morphology (arrow). Samples were dried on gold disks, overnight, and then sputtered with gold under vacuum prior to imaging. Pictures were taken with a Phillips XL30 ESEM.

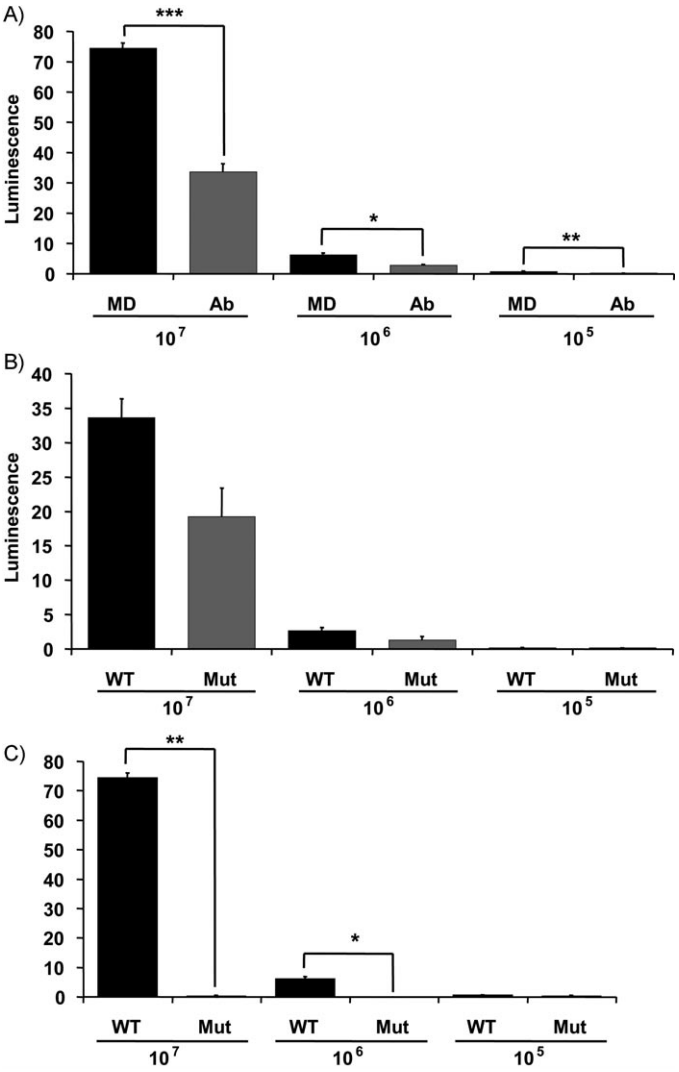


Figure 5. Comparison of antibody- and synthetic glycoconjugate-coated magnetic beads as capture reagents. A) A piliated *E. coli* strain (ORN178) was incubated with biotinylated bi-antennary mannose-conjugated (**MD**) magnetic beads or antibody (**Ab**) magnetic beads. The concentration range of *E. coli* was 10^7–10^5 CFU per well. The data are the mean ± standard errors of three experiments.***: $P < 0.0002$; *: $P < 0.04$; **: $P < 0.004$. **MD** magnetic beads have greater sensitivity than **Ab** magnetic beads at all three *E. coli* concentrations. B) Antibody assay with piliated *E. coli* (ORN178: WT) or nonpiliated (ORN208: Mut) strains. Both strains were incubated with **Ab** magnetic beads. The concentration range of *E. coli* was 10^7–10^5 CFU per well. The data are the means ± standard errors of three experiments. No significant ($P > 0.05$) selectivity for either strain was observed when using **Ab** beads. C) Glycoconjugate assay by using piliated *E. coli* (ORN178: WT) or nonpiliated (ORN208: Mut) strains. Both strains were incubated with biotinylated bi-antennary mannose-conjugated (**MD**) magnetic beads. The concentration range of *E. coli* was 10^7–10^5 CFU per well. Selectivity for the piliated strain was seen at higher *E. coli* concentrations. The data shown are the means ± standard errors of three experiments; **: $P < 0.0006$; *: $P < 0.02$.

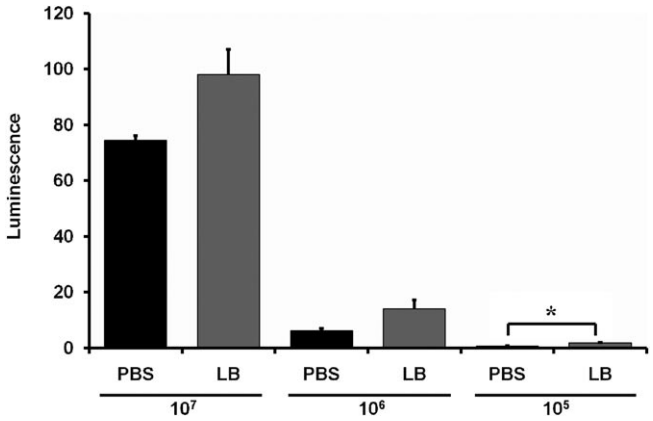


Figure 6. Effect of LB media on the sensitivity of the assay. After the capture of piliated *E. coli* (ORN178) with biotinylated bi-antennary mannose-conjugated (**MD**) magnetic beads, 10^7–10^5 *E. coli* were incubated with PBS at room temperature or LB media with glucose (2%) at 37 °C for 1 h. The data shown are the means ± standard errors of three experiments; *: $P < 0.03$ for the lowest concentration. Sensitivity can be increased with a preincubation step prior to measuring luminescence.

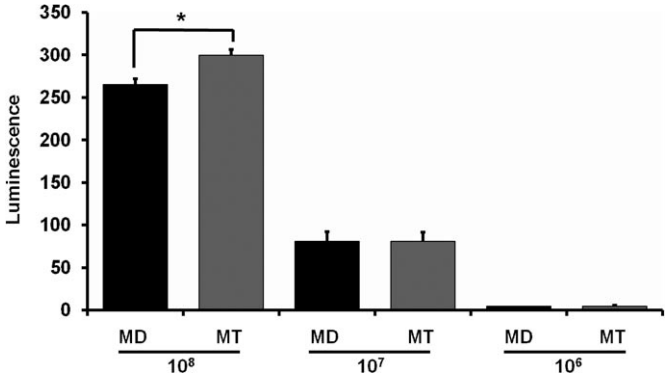


Figure 7. Limit of detection of piliated *E. coli* (ORN178) by using bi- and tetra-antennary magnetic beads. The concentration range of *E. coli* was 10^8–10^6 CFU per well. The data shown are the means ± standard errors of three experiments; *: $P < 0.03$ at the highest concentration of *E. coli*, 1×10^8 CFU per well. The effect of the increased valency of the mannose tetramer bead is observed only at the highest *E. coli* concentration.

Screening of pathogenic *E. coli,* including O157:H7, for glycoconjugate binding specificities

Binding to mannose is commonly associated with nonpathogenic strains of *E. coli*, however pathogenic strains also recognize mannose in addition to other glycans, which enable them to colonize sites outside the intestine. We used the panel of glycans (Figure 1) to examine the binding profiles of different pathogenic strains of *E. coli* (listed in the Supporting Information). Strain J96 and CFT073 are urinary tract pathogens isolated from individuals with pyelonephritis.[42,43] Strain J96-pilE is a mutant of J96 that lacks the ability to produce mannose-binding pili.[44] Strain B41 produces K99 pili, which are associated with the ability to colonize the intestinal tract of calves and pigs.[45] Strain PT22ΔTox was derived from a clinical isolate of *E. coli* O157:H7.[46]

The results are shown in Figure 8. ORN178, the two pyelonephritis strains, and the K99 strain showed significant binding to both mannose-bead variants, but not to lactose or sialic acid derivatives. As expected, the pilus mutants ORN208 and J96-PilE did not bind to either **MD** or **MT**. *E. coli* O157:H7 also failed to bind to the mannose-containing compounds; this is consistent with reports that O157:H7 strains are natural mutants for mannose pilus expression. Binding to the **LD**-, **SD**-,

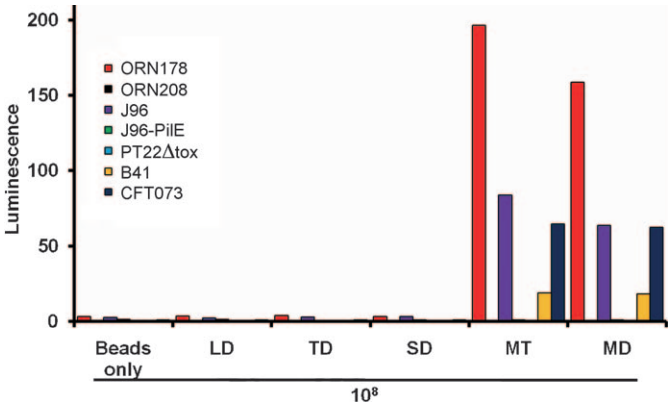


Figure 8. Screening studies. The two laboratory strains of *E. coli* (ORN178, ORN208), a clinical isolate of *E. coli* (J96, UPEC, and a nonpiliated derivative, J96-PilE), a human STEC isolate of O157:H7 (PT22Δtox), a calf pathogen B41 expressing K99 pili, and UPEC strain CFT073 were incubated with five different glycan-bead combinations (**MD**, **MT**, **LD**, **SD**, and **TD**). Strains ORN178, J96, B41, and CFT073 showed significant binding to both mannose-bead variants.

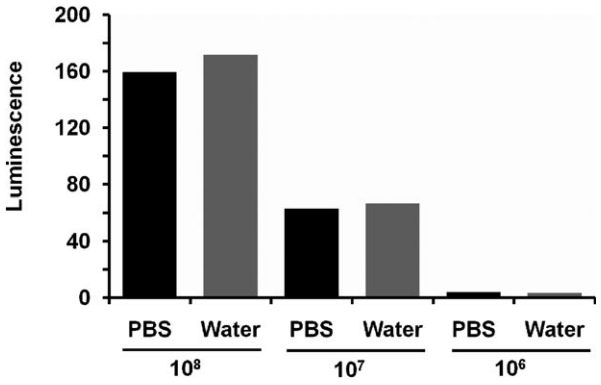


Figure 9. Detection of *E. coli* in real samples. PBS was spiked with either piliated *E. coli* (ORN178) or water from a local pond and incubated with biotinylated bi-antennary mannose-conjugated magnetic beads (**MD**). *E. coli* dilutions ranged from 10^8–10^6 CFU per well. The matrix of a real water sample did not reduce the sensitivity of this assay.

and **TD**-coated magnetic beads was not detected. This is in contrast to reports that suggest that *E. coli* O157:H7 binds to sialic acid.[47] However, it must be noted that natural ovalbumin, a heavily sialylated protein, and not synthetic glycoconjugates, were used in the previous study. We are currently developing a glycan microarray platform that uses a combination of synthetic glycoconjugates to develop a fingerprint for all strains.

Stability of synthetic glycoconjugates

The stability of glycoconjugates is an important factor if these affinity reagents are to be used in hand-held and environmental biosensors. The synthetic ligand was dissolved in water (2 mg mL^{-1}) and incubated at 50 °C for 48 h. The NMR spectra exhibited no apparent change and more importantly, the capturing ability of the ligands did not diminish. We also kept the ligands for over six months in solution or in a solid form at room temperature (ca. 25 °C) and observed no loss of biological function (data not shown).

Ability of synthetic glycoconjugates to capture bacteria from real samples

Interference is a significant problem in real samples. Indeed, high affinity reagents can be extremely cross-reactive and can capture extraneous material in addition to the analyte of interest. To this end, we used water from a local pond, spiked it with different concentrations of ORN178, and evaluated the ability of **MD** to capture bacteria. As seen in Figure 9, the matrix of a real water sample does not reduce the sensitivity of this assay. This is particularly exciting because these glycoconjugates can bind and detect bacteria from environmental samples or can be used in combination with other detection technologies as a pre-enrichment step.

Conclusions

The development of nonantibody based recognition elements is a critical research endeavor for diagnostic applications, in particular for environmental and point-of-care diagnostics. Here, we have shown that it is possible to develop glycoconjugates that exhibit antibody-like selectivity and sensitivity. This modular synthetic strategy will allow us to develop a library of chemically defined glycoconjugates that can be used to understand the basic biology of glycans beyond the biosensing aspect, which was the major focus of this report. The synthesis is extremely versatile, and any azide-containing biomolecule can be attached to the scaffold. Biotin is very attractive, as it provides a handle for coupling to any streptavidin-coated matrix, such as magnetic iron oxide nanoparticles,[48] gold silica nanoshells,[49] and quantum dots.[50] A head-to-head comparison of "gold standard" antibodies and synthetic glycoconjugate-coated magnetic beads revealed the power of glycans in biosensing. Using a magnetic bead based luminescence assay, we have demonstrated that synthetic reagent-coated magnetic beads outperform antibody-coated magnetic beads in sensitivity and selectivity. The molecules are also very stable, inexpensive, and can capture pathogens from stagnant water. With these recognition elements, higher sensitivity could be achieved by using a more sensitive technique or a combination of transduction methods, such as spectroelectrochemical sensors.[51] The results presented here are expected to lead to an increased interest in developing glycoconjugate-based high affinity reagents for diagnostics.

Experimental Section

Synthesis: The synthesis of the five glycoconjugates is given in the Supporting Information.

Microbiology

Streptavidin-coated magnetic beads (Dynabeads M-280) were purchased from Invitrogen. Biotinylated anti-*E. coli* antibody (Rabbit IgG polycolonal antibody) was purchased from GenWay Biotech,

Inc. (San Diego, CA, USA). BacTiter-Glo® was purchased from Promega.

Bacterial cultures: L-Agar plates were streaked for isolation and incubated, overnight, at 37 °C. A single colony from each plate was used to inoculate LB medium (10 mL), and the bacteria were grown statically at 37 °C, overnight, to an OD_{600} of ~1.0 (10^8 cells mL^{-1}). Appropriate dilutions were made in phosphate buffered saline (PBS, pH 7.4) prior to incubation with conjugated magnetic beads or for the different assays.

Conjugation of biotinylated ligands to magnetic beads: Aliquots of magnetic beads (1 mL, 10 mg mL^{-1}) were washed three times with PBS buffer (1 mL). The beads were incubated with biotinylated ligands (either 10^3 pmol mg^{-1} of bead or 100 μg antibody per mg of bead) on an orbital shaker for 30 min. The tubes were then placed over a magnet, the supernatant was removed, and the beads were washed three times with PBS (1 mL) to ensure complete removal of unbound glycan or antibody.

Agglutination assay with MD with ORN178 and ORN208: *E. coli* strains ORN178 and ORN208 were diluted (1×10^8 CFU mL^{-1}) and 100 μL was added to wells of a 96-well plate. The MD bead solution (5 μL) was injected. Pictures were taken by using a Premier digital 10X microscope eyepiece inserted into a Nikon TMS microscope before and ten minutes after addition of magnetic beads.

ESEM images: *E. coli* strains ORN178 and ORN208 (10^7 CFU mL^{-1}) in PBS (pH 7.4) were incubated with mannose dimer beads (25 μL) at room temperature for 1 h on the orbital shaker. Within 10 min, visible aggregates formed in the ORN178 tube. The tubes were placed over a magnet for 5 min, the PBS was carefully removed, the tubes were washed with sterile deionized water (1 mL), vortexed, and suspended in water (1 mL) to generate the final SEM samples. Droplets from each sample were placed on a gold disc and allowed to air-dry, overnight. The gold disc was sputtered with gold in vacuo. Images were captured by using a Phillips XL30 ESEM.

Comparison of antibody- and synthetic glycoconjugate-coated magnetic beads as capture reagents: Diluted *E. coli* strains ORN178 and ORN208 (1×10^7–1×10^5 CFU per well) in 1.5 mL Eppendorf tubes were incubated with either **MD** beads or **Ab** beads (25 μL) at room temperature for 1 h on an orbital shaker. Visible agglutination was seen with the mannose dimer-bead and the ORN178 at 1×10^7 CFU per well. The tubes were then placed over a magnet for 5 min. The supernatant was carefully removed, and the tubes were washed with PBS (1 mL), and suspended in PBS (100 μL). The samples were transferred to a 96-well microtiter plate designed for use in a luminometer (Thermo Labsystems Luminoskan Ascent 96-microwell plate reader). Controls included unconjugated beads in PBS and LB. A multichannel pipette was used to inject BacTiter-Glo reagent (100 μL) into each well. The plate was immediately placed in the Luminometer (Labsystems Luminoskan Ascent). A 5 min shake step was followed by a 5 min incubation step at room temperature prior to measuring the luminescence. Each assay was repeated in triplicate on three different days. The Student's T test was used to determine statistical significance. To enhance sensitivity, identical samples were prepared, but one set was suspended in LB media (100 μL) containing glucose (2.0%). These tubes were incubated at 37 °C for 1 h prior to determining the luminescence.

Capturing ability of bi- and tetra-antennary glycoconjugates: Diluted *E. coli* strain ORN178 (1×10^7–1×10^5 CFU per well) in 1.5 mL Eppendorf tubes was incubated with either **MD** or **MT** beads (25 μL) and processed as described previously.

Screening of pathogenic *E. coli*, including O157:H7, for glycoconjugate binding specificities: ORN178, ORN208, J96, J96 pilE, CFT073, B41, PT22ΔTox (1×10^8 CFU) were incubated with glycoconjugated beads (25 μL, 1.5×10^7 of **MD**, **MT**, **LD**, **SD**, **TD**) or unconjugated beads (blank) and processed as described previously.

Ability of synthetic glycoconjugates to capture bacteria from real samples: *E. coli* strain ORN178 (1×10^8–1×10^6 CFU) was suspended in PBS buffer (1 mL) or water from a local pond in 1.5 mL Eppendorf tubes. **MD** or unconjugated (blank) beads (25 μL) were added and processed as described previously. A second control experiment involved the use of water only from a local pond (Burnet Woods, across University of Cincinnati Main Campus).

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