# Detection of Bacteria with Carbohydrate-Functionalized Fluorescent Polymers

Matthew D. Disney,[†] Juan Zheng,[‡] Timothy M. Swager,[‡] and Peter H. Seeberger*,[†]

*Contribution from the Laboratory for Organic Chemistry, Swiss Federal Institute of Technology Zürich, ETH Hönggerberg HCI F315 Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland, and Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139*

Received April 9, 2004; E-mail: seeberger@org.chem.ethz.ch

***Abstract:*** Many pathogens that infect humans use cell surface carbohydrates as receptors to facilitate cell−cell adhesion. The hallmark of these interactions is their multivalency, or the simultaneous occurrence of multiple interactions. We have used a carbohydrate-functionalized fluorescent polymer, which displays many carbohydrate ligands on a single polymer chain, to allow for multivalent detection of pathogens. Incubation of a mannose-functionalized polymer with *Escherichia coli* yields brightly fluorescent aggregates of bacteria. These results show that carbohydrate-functionalized fluorescent polymers are a versatile detection method for bacteria. Future design of detectors for other pathogens only requires information on the carbohydrates bound by the organisms, which has been exhaustively reported in the literature.

## Introduction

Enterohemorragic *Escherichia coli*-contaminated foods are a major cause of infection outbreaks with serious consequences. One of the largest outbreaks occurred in Japan in 1996, where over 10 000 people were infected and 11 died.[1] One potential reason for this outbreak reaching these catastrophic proportions was the absence of testing food for contaminants before public consumption. Current methods that are used clinically for the detection of pathogenic bacteria, such as *E. coli*, rely on selective growth of the bacteria from a contaminated sample, which can take several days.[2] More recently, faster methods have been developed that include pathogen recognition by fluorescently labeled antibodies,[3] DNA probes,[4] or bacteriophages.[5] While fluorescent conjugated polymers have found use in a variety of biological sensing applications,[6] such as recognition of proteins by electrostatic interactions[7] and detection of pathogens by DNA hybridization,[8] detection schemes for cells have not been reported.

In many cases, pathogens bind to carbohydrates displayed on the cells they infect. A series of carbohydrate−pathogen interactions have been described: *E. coli* binds to mannose, influenza virus binds to sialic acid, etc.[9] The interactions of pathogens to cell surface carbohydrates are often multivalent, which results in higher binding avidity compared to monovalent binding.[10] Polymer-based detection methods for cells have the advantage of simulating multiple interactions through the display of many ligands on a single polymer chain. Here, we report a carbohydrate-functionalized poly(*p*-phenylene ethynylene) (PPE) that can be used for detection of *E. coli* by multivalent interactions. In contrast to previous examples of sugar-containing PPEs,[11] the polymer is functionalized after polymerization and provides a versatile scaffold for the rapid attachment of a variety of different carbohydrates.

## Results and Discussion

**Synthesis of Carbohydrate-Functionalized Fluorescent Polymers.** Coupling of the 2′-aminoethyl mannoside and galactoside[12] to the PPE was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and

† Swiss Federal Institute of Technology Zürich.
‡ Massachusetts Institute of Technology.
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**Figure 1.** Structures of the polymers used in this study.
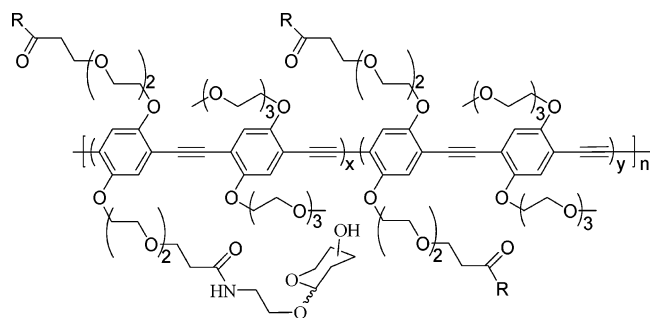
**1** R=OH; x : y = 0 : 1

**2a** R = OH or NH(CH$_2$)$_2$OH; x : y = 1 : 1
sugar = mannose

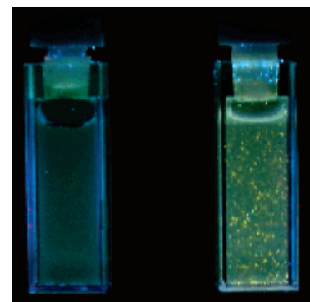**2b** R = OH or NH(CH$_2$)$_2$OH; x : y = 1 : 1
sugar = galactose
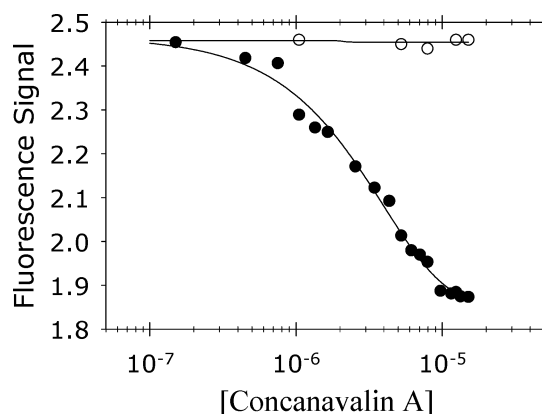


**Figure 2.** Plot of the normalized fluorescence signal at 512 nm from addition of Alexa Fluor 594-labeled Con A to a solution containing mannose-functionalized polymer 2a (●) or galactose-functionalized polymer 2b (○).



**Figure 3.** Visualization of mutant, left, and mannose-binding, right, *E. coli* strains after incubation with mannosylated polymer **2a**. Approximately 10 μg of polymer was incubated with 1.0 OD$_{600}$ of cells. Cells were imaged under a transilluminator.

*N,N′*-diisopropylethylamine for 16−24 h. This was followed by quenching of unreacted succinimide esters via addition of excess ethanolamine. Uncoupled reagents were removed by dialysis of the reaction mixture against water for 2 days. A phenol sulfuric acid test[13] for carbohydrate loading showed that typically 25% of the reactive sites on the polymer were functionalized with glycosides.

**Fluorescence Resonance Energy Transfer (FRET) To Test Lectin−PPE Interactions.** To ensure that the carbohydrate moieties conjugated to the polymer retained their ability to interact with carbohydrate-binding lectins, a FRET experiment was carried out between Alexa Fluor 594-labeled concanavalin A (Con A, a mannose-binding protein[14]) and each of the sugar-functionalized PPEs (Figure 1). Titration of labeled Con A into a solution of mannose-functionalized polymer showed a concentration-dependent decrease in fluorescence signal (Figure 2). Experiments with galactose-functionalized polymer showed no fluorescence change, as expected. Thus, mannose-binding lectins interact with mannose displayed on the polymer without affecting binding selectivity. Furthermore, the polymer does not exhibit any nonspecific binding to Con A.

**Bacterial Detection Using Carbohydrate-Functionalized PPEs.** Two bacterial strains that differ only in their mannose-

binding properties were used to assess whether the mannose-functionalized PPE **2a** can fluorescently stain *E. coli*. In addition to a strain that binds to mannose, a second strain was used where its FimH protein was mutated to abolish mannose binding.[15] The nonfunctionalized polymer **1**, the mannosylated polymer **2a**, and 2′-fluorescein aminoethyl mannoside were individually incubated with these bacterial strains. After incubating a 1-mL bacterial suspension at an OD$_{600}$ of 1.0 (10$^8$ cells) with the appropriate polymer or dye-labeled mannose for 30 min, we centrifuged the suspensions to pellet the cells. The supernatant solution containing unbound polymer or dye-labeled mannose was discarded, and the cells were washed twice with phosphate-buffered saline (PBS, pH 7.2). The bacteria were then resuspended in PBS. Neither polymer **1** nor the 2′-fluorescein aminoethyl mannoside appear to bind to either *E. coli* strain. The mannose-functionalized polymer, **2a**, however, imparted a strong fluorescent label to mannose-binding *E. coli* (Figure 3) that is not removed even upon separation and rinsing. The resuspended rinsed non-mannose-binding *E. coli* gave no polymer fluorescence after incubation with **2a**.

Binding of mannose- and galactose-functionalized polymers to bacteria were microscopically imaged. After incubation with **2a**, the mutant bacteria remain as individual cells that do not bind to polymer (Figure 4A), while the mannose-binding bacteria form clusters with fluorescent centers where the polymer is bound to many cells (Figure 4B). These brightly fluorescent clusters are formed by thirty to several thousand bacteria (Figures 3, 4B, and 4C). The larger clusters have the strongest fluorescence signal, while single cells in the culture exhibited little fluorescence. Though aggregation of Jurkat cells has been previously observed with Con A attached to mannose-functionalized ROMP polymers,[16] such direct polymer cell clustering has not been reported with the much smaller *E. coli* nor has it been used for detection purposes. Additionally, the fluorescence emission spectrum of the polymer in the bacterial clusters exhibited a more red-shifted and aggregated behavior (increased emission at 550 nm) than spectra in PBS solution (Figure 4D). This is consistent with increased π-stacking interactions between the polymer strands as they are brought into closer proximity by the bacteria. After incubation with **2b**, neither mutant nor the mannose-binding bacteria were fluorescently stained; this is expected since *E. coli* does not bind to galactose (see Supporting Information).

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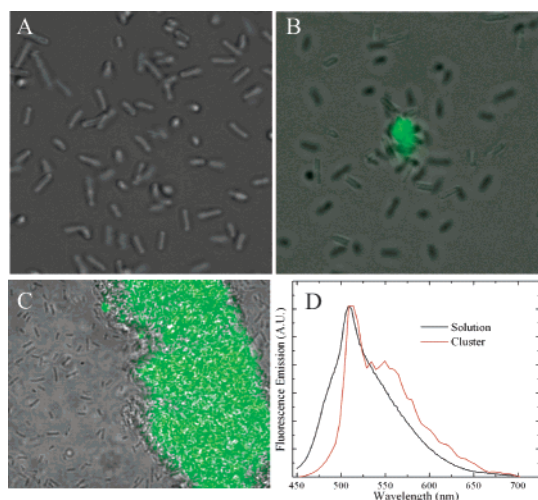
**Figure 4.** Laser scanning confocal microscopy image of (A) mutant *E. coli* that does not bind to **2a**. Individual cells observed with no aggregation. (B) A fluorescent bacterial aggregate due to multivalent interactions between the mannose-binding bacterial pili and **2a** (superimposed fluorescence and transmitted light images). (C) Fluorescence microscopy image of a large fluorescent bacterial cluster. (D) Conventional fluorescence spectra of **2a** in PBS (black) and normalized fluorescence spectra of a bacterial cluster obtained using confocal microscopy (red).
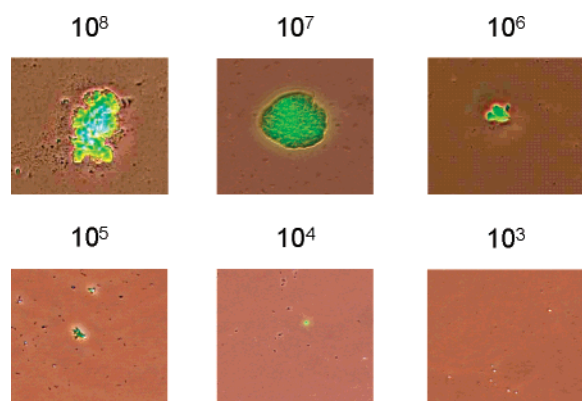


**Figure 5.** The detection limit for the staining of the mannose-binding *E. coli* by **2a**. The number of bacteria incubated with the polymer-containing solution is indicated above each image.

Serially diluted solutions of mannose-binding *E. coli* were incubated with **2a**, washed to remove unbound polymer, and imaged using fluorescence microscopy to determine the detection limit. Results show that fluorescently stained clusters of cells can be observed with as little as $10^4$ bacteria (Figure 5).[2] This is similar to the detection limit that is observed using fluorescently labeled antibodies. Furthermore, the number of cells that is present in the clusters decreases with diminishing cell numbers.

These results suggest that multivalent interactions are critical for detection, since the mannosylated PPE allowed for fluorescent detection of *E. coli* while 2′-fluorescein aminoethyl mannoside did not. The multivalent binding nature of **2a** was demonstrated by testing this polymer for inhibition of Con A-induced hemagglutination of sheep erythrocytes (Table 1). The concentration of mannose displayed by the PPE to inhibit hemagglutination was more than 500-fold less than that for the monomeric mannose derivatives, indicating that polymers bind Con A in a multivalent manner. The observed enhancement is similar to that reported with polymers prepared by ring-opening metathesis polymerization (ROMP).[17]

**Table 1.** Inhibition of Sheep Red Blood Cell Hemagglutination[a]

| compound | inhibiting dose, M |
| --- | --- |
| mannose | 0.02 |
| 2′-aminoethyl mannoside | 0.01 |
| 2′-fluorescein aminoethyl mannoside | 0.01 |
| mannose conjugated polymer **2a** | $16 \times 10^{-6}$ |
| nonfunctionalized polymer **1** | N.D.[b] |

[a] Inhibiting doses are the average of at least three independent measurements and are expressed in the concentration of mannose residues. [b] No inhibition was observed. The errors are ±50%.
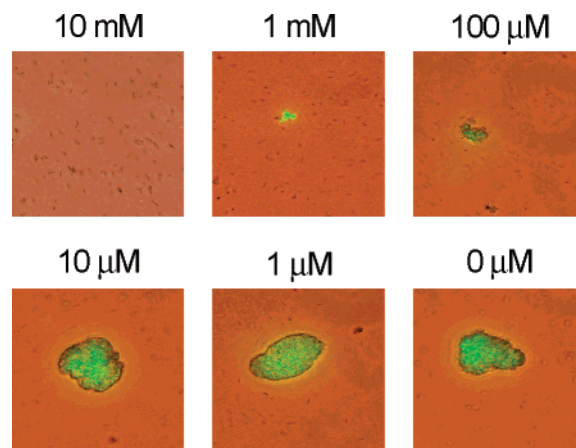


**Figure 6.** Competition of **2a** binding to mannose-binding *E. coli* by D-mannose. In this experiment, the concentration of mannose displayed by the PPE is $2.9 \times 10^{-9}$, and the concentration of D-mannose varied and is indicated above each microscopic image.

Competition experiments were also completed to determine the concentration of D-mannose that inhibited the binding of **2a** to the mannose-binding *E. coli*. Experiments were completed with 10 $\mu$g of **2a** or $2.9 \times 10^{-9}$ mol of mannose conjugated to the PPE and increasing concentrations of D-mannose. Results show that a 10 mM concentration of D-mannose was needed to completely inhibit binding of **2a** to *E. coli*. At concentrations of mannose that are less than 10 $\mu$M, the size of the bacterial aggregates is not affected (Figure 6). The enhancement in binding due to the multivalency of **2a** is $3.5 \times 10^4$-fold, indicating significant enhancement in binding due to multivalency.

**Insights into Future Development of Biosensors.** A catalog of carbohydrate−pathogen interactions is known in the literature.[10] Some of these interactions, however, are not specific for one type of pathogen; an example of this is the cross-reactivity of mannose toward *Salmonella enterica* and *E. coli*. The limitation of having ligands with imperfect selectivities can be overcome through the use of cross-reactive sensor analysis.[18] In these experiments, the presence of a ligand is determined through the binding of many different analytes; such a detection scheme is used by the nose. Thus, many different carbohydrates can be coupled to polymers and analyzed in parallel, perhaps in a 96-well plate format. This should allow detection of the presence of a single or multiple pathogens within complex mixtures.

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## Conclusions

A new method for fluorescent detection of bacteria based on water soluble fluorescent-conjugated polymers has been developed. Glycosides displayed on the surface of the polymers retain their ability to interact with known carbohydrate-binding lectins. Incubation of the polymers with *E. coli* shows that the polymers bind to the bacteria and yield brightly fluorescent cell clusters. This aggregation is due to multivalent interactions between the mannosylated polymer and mannose receptors located on the bacterial pili, which was corroborated by microscopy, hemagglutination, and competitive binding experiments. This multivalency and resulting cell aggregation is essential for detection. In contrast to methods for pathogen detection that use selective growth in liquid media or on plates, which can take several days, carbohydrate-functionalized PPEs can detect the presence of a pathogen in as little as 10 to 15 min. The preference of different bacteria to bind to specific carbohydrates allows the potential sensing of a range of pathogens such as cholera in water and other sources. Work in this area is underway in our laboratories and will be reported in due course.

**Supporting Information Available:** General methods for polymer synthesis and other experimental details are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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