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Development of Microfluidic Chips for Heterogeneous Receptor–Ligand Interaction Studies

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A simple microfluidic-based technique to quantitate the binding affinity between the glycopeptide antibiotics teicoplanin from *Actinoplanes teicomyceticus* and vancomycin from *Streptomyces orientalis* and 5-carboxyfluorescein-D-Ala-D-Ala-D-Ala (5-FAM-(DA)₃) is described. In this work, (3-aminopropyl)triethoxysilane is used to modify the surfaces of a series of microchannels, and each channel is subsequently exposed to a solution of antibiotic for a few minutes. The antibiotic is retained after washing through electrostatic interactions, and the series of channels are subsequently exposed to an increasing concentration of 5-FAM-(DA)₃ followed by washing to exclude any nonspecific binding. The extent of fluorescence is quantified using a microscope fitted with a CCD camera. The binding constants for the interaction of teicoplanin and vancomycin with the fluorescent peptide were determined to be $6.03 \pm 0.97 \times 10^4$ and $4.93 \pm 1.13 \times 10^4 \text{ M}^{-1}$, respectively, in good agreement with previous data. The ease of quantifying the extent of interaction in this microchip technique may prove powerful for exploration of a myriad of receptor–ligand pairs.

The understanding of interactions between receptors and ligands in biological systems holds important information on the initiation, progression, and harmful effects of human diseases, such as AIDS/HIV, Alzheimer's, Parkinson's, and cancer. To develop new drugs and screen potential candidates, there is a great demand for analytical technologies that can rapidly and reliably investigate biological interactions by measuring affinity parameters. One such technique that has proven successful in determining binding parameters between a host of receptor–ligand systems^{1–12} is affinity capillary electrophoresis (ACE) both in open

tubular form and in a microchip format.^{13–17} In one form of ACE, the binding constant (K_b) of a receptor–ligand pair is determined by measuring the change in electrophoretic mobility of the receptor relative to an internal marker(s) on increasing the concentration of a charged ligand. Subsequent analysis using a Scatchard model or other type of analysis yields a value for K_b . Uncharged ligands can also be assessed for their binding to a receptor but require a competitive type of assay be used in the ACE technique.^{18–20}

Microfluidic heterogeneous assays for receptor–ligand binding studies (mostly immunoassays) have several advantages over electrophoresis-based microfluidic assays, such as high sensitivity and selectivity and simple separation of complexed and uncomplexed molecules.²¹ The receptor (or ligand) molecules are immobilized on a solid support through adsorption,^{22–24} covalent binding,^{25–27} or

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microcontact printing.^{28–30} There are two types of solid supports used in microfluidic heterogeneous assays, micro/nanobeads^{31,32} and the wall surfaces of the microchannel.^{33,34} In this study, we focused on the latter. Our work on the use of magnetic microbeads in binding assays was reported earlier.³⁵ Silicon, quartz, and glass are the first materials used to fabricate the microchannels for heterogeneous assays because of their well-studied physical and chemical properties and established fabrication protocols. However, silicon, quartz, and glass fabrication requires sophisticated facilities, such as a mask aligner, and sometimes nasty chemicals such as hydrofluoric acid. Polymers, such as PMMA,^{36–38} polyester,^{39–41} and polystyrene,^{42,43} are now popular materials for microchannel fabrication because of their low cost, optical and mechanical properties, and simpler fabrication processes (hot embossing, injection molding, and replication, etc.).^{44,45} Among these polymers, poly(dimethylsiloxane) (PDMS) has received a great deal of attention and has been widely used to fabricate microstructures for many biological and chemical studies. It has a low cost and is stable and optically transparent. Various microfluidic components, e.g., pumps and valves, can be easily produced using soft lithography.^{46,47} However, the hydrophobicity of PDMS leads to nonspecific adsorption of analytes (such as proteins), resulting in deteriorated assay performance due to a lower signal-to-noise ratio. To address this issue, different strategies of surface modification, e.g., oxygen plasma and surface silanization, have been applied to PDMS microfluidic devices to tailor the surface properties for desired applications.^{21,48} (3-Aminopropyl)triethoxysilane (APTES) has been used to modify surfaces of microchannels,^{49–51} and its free amine groups on the treated surfaces offer possibilities

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Table 1

ref	binding constants (K_b , 10^{-4} M^{-1})	
	teicoplanin	vancomycin
this work	6.03 ± 0.97	4.93 ± 1.13
6	2.11	1.53
18	12.23	1.91
19	15.32	1.14

to immobilize a wide spectrum of molecules, such as antigens and antibodies, for biological assays.

Herein, we describe a simple microfluidic chip setup for heterogeneous receptor–ligand studies. The microchip was fabricated in PDMS using soft lithography and treated with APTES at room temperature for 30 min to immobilize the glycopeptide antibiotics teicoplanin and vancomycin. Fluorescence microscopy was used to study the interactions of 5-carboxyfluorescein-D-Ala-D-Ala-D-Ala (5-FAM-(DA)₃) with the two antibiotics which were immobilized on the microchannel walls via APTES surface modification. The measured values for K_b are $6.03 \pm 0.97 \times 10^4$ and $4.93 \pm 1.13 \times 10^4 \text{ M}^{-1}$ for teicoplanin and vancomycin, respectively, which are in good agreement with literature values measured on commercial CE systems (Table 1).^{6,18,19}

EXPERIMENTAL SECTION

Materials. 5-FAM-(DA)₃ (**1**) was synthesized by Anaspec Inc. (San Jose, CA). APTES (A3648) and vancomycin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Negative-type photoresist SU-8 2025 and developer were obtained from Microchem (Newton, MA). PDMS oligomer and its cross-linking agent Sylgard 184 were purchased from Dow Corning (Midland, MI). Teicoplanin was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). AggRecetin (Ristocetin) was obtained from Bio/Data Corp. (Horsham, PA).

Microchip Fabrication. The mold master was made using the negative photoresist, SU-8 2025, on a 6 in. silicon wafer with a high-resolution transparency mask (typically 3386, 8000 or 20000 dpi). The channel depth ($\sim 75 \mu\text{m}$ for our devices) was controlled by the spin-coating speed. The PDMS prepolymer and its curing agent were mixed at the weight ratio of 10:1. The mixture was degassed in a vacuum chamber and then poured onto the mold master. The microchip was cured at 75°C for 2 h. There were 10 microchannels on each fabricated PDMS chip (Figure 1). The access ports for each microchannel ($25 \mu\text{m}$ in diameter) were made with a manual puncher (Technical Innovations, Inc., Brazoria, TX). The PDMS replica was thoroughly washed with ethanol and exposed to oxygen plasma to seal the chip to a clean glass slide (catalog number 48300-025, VWR International, West Chester, PA).

Surface Modification of Microchannels. APTES was prepared as a 10% ethanol solution. For silanization, the PDMS microchannels were filled with the APTES/ethanol solution and incubated for 30 min at room temperature. Upon completion, the treated microchannels were washed with ethanol and deionized water. For binding experiments, eight of the treated microchannels were loaded

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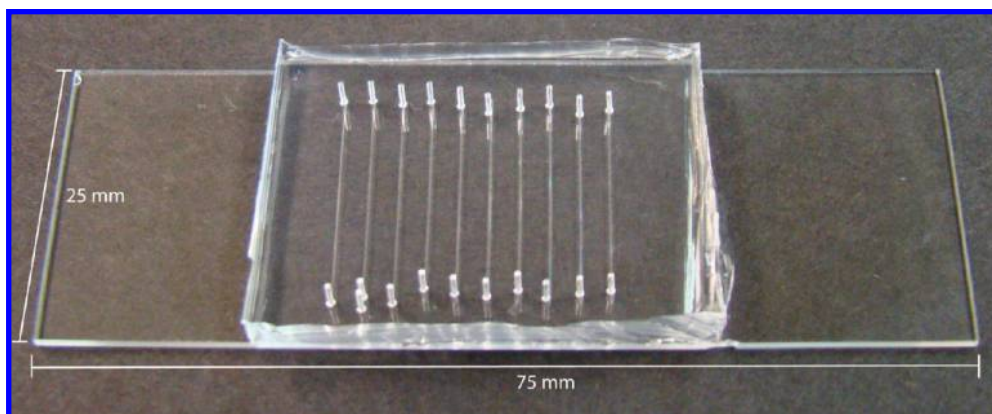


Figure 1. PDMS microfluidic chip.

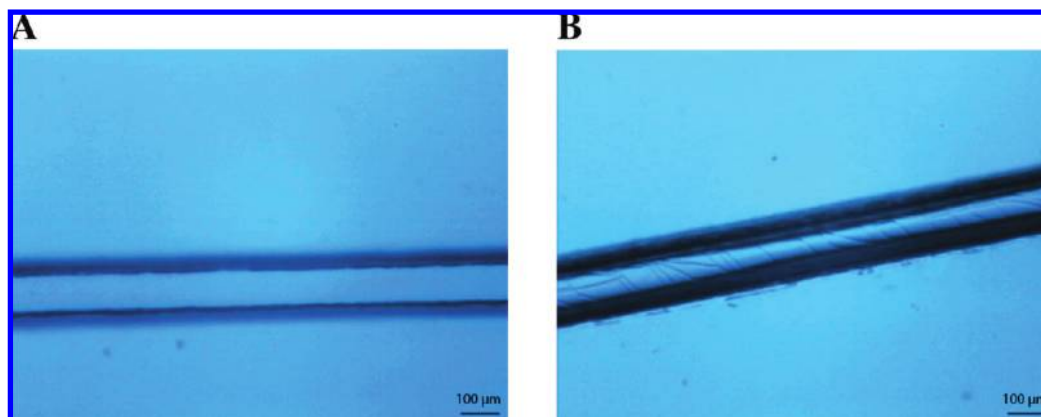


Figure 2. Optical micrographs of the test microfluidic device: (a) control untreated microchannel, (b) APTES-treated microchannel.

with teicoplanin or vancomycin solutions ($30 \mu\text{M}$ in phosphate-buffered solution (PBS), B9431 from Sigma-Aldrich Inc.) and incubated for 30 min at room temperature. Two control channels were filled with bovine serum albumin (BSA) ($50 \mu\text{M}$). After incubation, all channels were washed with deionized water and then incubated with a PBS solution containing 3% BSA for 30 min at room temperature. All of the channels were again washed with PBS, filled with varying concentrations of **1** ($7\text{--}454 \mu\text{M}$), and allowed to incubate for another 30 min at room temperature. After incubation, the channels were washed with PBS and were ready for imaging.

Data Acquisition and Processing. Fluorescence data collection was performed on an inverted epifluorescence microscope (Nikon Eclipse-2000U) equipped with a CCD camera (IN1830, Diagnostic Instruments, Inc., Sterling Heights, MI). A $40\times$ objective was used to focus the excitation and emission light (488 and 520 nm, respectively). Image acquisition (16 bit grayscale) was performed via the CCD camera controlled by SPOT software. The exposure time for each shot was 400 ms. The intensity data were then extracted with ImageJ, the background subtracted, and plotted against corresponding concentrations of **1**. The binding isotherms were fitted with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) to the Langmuir isotherm to determine the binding constants.⁵²

RESULTS AND DISCUSSION

Effects of APTES Treatment. The incubation time for APTES treatment was determined to be 30 min for the microchannels used

($100 \mu\text{m}$ by $75 \mu\text{m}$ in the cross section). The microchannels become clogged if the incubation time exceeds 30 min. The coating process was performed at room temperature instead of an elevated temperature (e.g., 80°C) in an oven, and good coating results were observed (Figure 2). We also performed pressure tests on both APTES-treated and untreated PDMS microchannels to compare the highest pressure before leakage set in. It was found that the APTES-treated microchannels can withstand 5 times the pressure of untreated channels. The APTES coating increased the mechanical strength of the PDMS microchannels, which can be exploited in applications involving high pressure, such as liquid chromatography.

Binding Studies. The surfaces of APTES-coated microchannels have free amine groups available for subsequent derivatization. Amine coupling is the most commonly used reaction to covalently immobilize molecules via amide bond formation with carboxyl-terminated species. To start the reaction, carboxylic groups are usually first activated with a coupling agent, for example, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), and then incubated with amine groups to form the amide bond.⁵¹ In this work, the two model antibiotics carry free carboxylic groups available for amine coupling. We directly incubated the antibiotic solutions with APTES-treated microchannels without EDC and NHS present in the solution at room temperature and found that the antibiotic molecules were immobilized under these conditions. As shown in Figure 3, the microchannels with immobilized teicoplanin and vancomycin showed fluorescence after incubation with **1**. To verify this observation, we tried a third antibiotic, ristocetin, which has a structure similar to those of the

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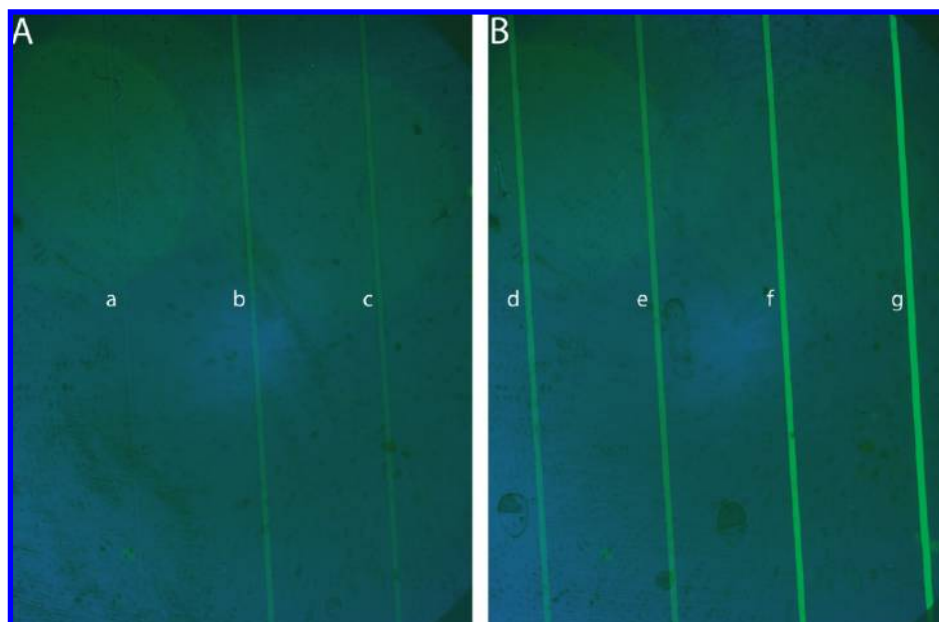


Figure 3. Fluorescence inside the microfluidic device for low to high concentrations of **1**, where $a = 7$, $b = 14$, $c = 28.38$, $d = 56.75$, $e = 113.5$, $f = 227$, and $g = 454 \mu\text{M}$, respectively. The images were taken at an exposure time of 400 ms to avoid photobleaching and then analyzed to determine the fluorescence intensity at each concentration.

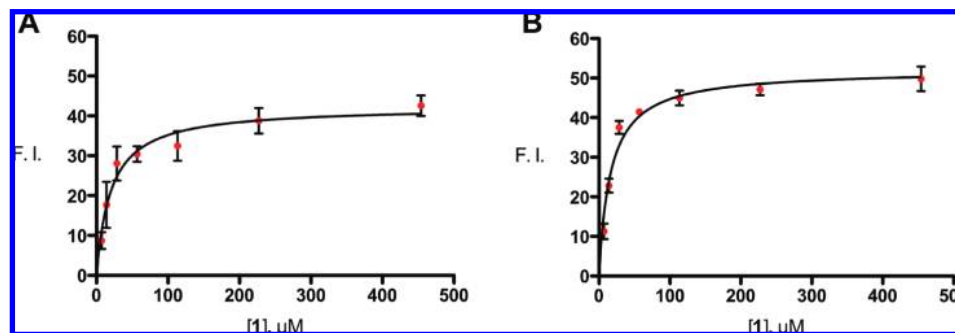


Figure 4. (A) Binding isotherm for vancomycin with **1** ($n = 8$). (B) Binding isotherm for teicoplanin with **1** ($n = 6$).

two model ones except it has no free carboxylic group. We found that, after incubation with **1**, the microchannels treated with ristocetin did not show any fluorescence, which demonstrated that ristocetin was not immobilized as the other two antibiotics under the same reaction conditions. We speculate that the immobilization of teicoplanin and vancomycin onto the microchannel surface results from the electrostatic attraction between the positively charged amine group on the microchannel surface and the negatively charged carboxyl groups in the PBS buffer (pH 7.4).

The binding isotherms (Figure 4) were obtained by extracting fluorescence intensity data from images of the microchannels with antibiotics incubated with **1** at different concentrations ranging from 0 to 454 μM . The binding constants were determined to be $6.03 \pm 0.97 \times 10^4$ and $4.93 \pm 1.13 \times 10^4 \text{ M}^{-1}$ for teicoplanin and vancomycin, respectively, which are in good agreement with literature values.^{6,18,19}

CONCLUSIONS

We have demonstrated a simple microfluidic-chip-based assay to quantitate the extent of interaction between a receptor immobilized onto microchannel surfaces and a fluorescent ligand. All the steps of surface modification and molecule immobilization were carried

out by incubating reagents at room temperature. The binding constants for the model antibiotic–peptide pairs obtained are reproducible and in good agreement with the literature values. With further simplification in the signal detection method (currently fluorescence microscopy with a CCD camera), we believe that this microchip technique can be developed into a portable kit that includes a microchip, manual syringe, and a few chemical vials for general users to perform binding assays on relevant biomedical interactions in a physician's office or in defense-related scenarios involving pathogenic materials. Current work is focused on examining other receptor–ligand pairs that can be integrated into the microfluidic system.

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