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# Fabrication of Phospholipid Bilayer-Coated Microchannels for On-Chip Immunoassays

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**Herein we describe a new class of microfluidic immunoassays based upon solid supported lipid bilayers. Two-dimensionally fluid bilayer material, which can accommodate multivalent binding between surface-bound ligands and aqueous receptors, was coated on the surface of poly(dimethylsiloxane) microchannels. The bilayers contained dinitrophenyl (DNP)-conjugated lipids for binding with bivalent anti-DNP antibodies. Twelve independent data points of surface coverage versus bulk protein concentration could be made simultaneously by forming a linear array of channels and flowing fluorescently labeled antibodies into them. This enabled an entire binding curve to be obtained in a single experiment. The measured apparent binding constant for the DNP/anti-DNP system was 1.8 $\mu$M. The methodology for performing heterogeneous assays developed here not only produces rapid results but also requires much less protein than traditional procedures and eliminates some standard sources of experimental error.**

Multivalent ligand−receptor binding is a recurring motif in the biological sciences.[1,2] Such events are ubiquitous on cell surfaces where membrane-bound ligands and receptors undergo two-dimensional rearrangements to accommodate multiple interactions during a wide variety of processes. Typical examples include signal transduction and the attack of bacterial and viral pathogens on human hosts. Therefore, the formulation of strategies that accommodate multivalent binding should be an important step in the development of a new generation of biosensors and assays that more closely mimic in vivo attachment at cellular membranes.[3−8] An attractive platform for this purpose is the planar supported phospholipid bilayer (SLB).[9] SLBs, which can be formed on solid supports by vesicle fusion[10] and Langmuir−Blodgett methods,[11] maintain two-dimensional fluidity and, hence, allow membrane-associated ligands and receptors to undergo lateral rearrangements during multivalent binding. Mobility is retained because a thin water layer acting like a lubricant resides between the membrane and the underlying surface of this supramolecular architecture.[12−15] When the biomimetic surface is placed in a flow cell, a heterogeneous sensor platform is created over which proteins, viruses, bacteria, and other biological agents can be passed and their surface coverages monitored.[4,16−19]

Exploiting fluid bilayer-based platforms for sensor design could be made even more powerful by incorporating them into microfluidic networks for lab-on-a-chip assays.[20−22] Although exploitation of microfluidic technology to design immunoassays is still in its nascent stage, several groups have developed technology for homogeneous assays that exploit on-chip electrophoretic separation.[23−28] These investigators demonstrated that a microfluidic-based approach allows high-throughput, small sample volumes, and ease of automation. In comparison to homogeneous assays, less work has been done with heterogeneous microfluidic immunoassays. This is unfortunate as heterogeneous platforms have the added advantage of clear separation of bound and unbound species. One of the few heterogeneous examples is from work by Sato et al., in which an immunosorbent assay was developed that exploits polystyrene microbeads embedded inside lithographically patterned microchannels.[29] In these experiments, antigens

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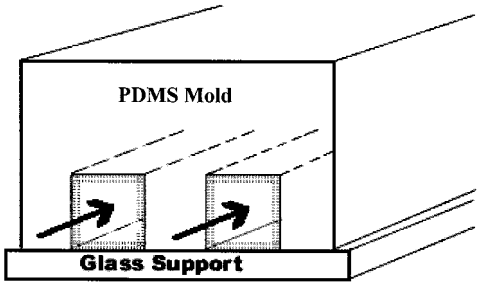


Figure 1. Schematic diagram of bilayer-coated PDMS microchannels on a planar glass substrate. The bilayer coats both the glass and polymer surfaces. Protein solutions can then be injected into the channels as indicated by the arrows.

were fixed to the beads and the binding of antibodies with covalently attached colloidal gold was quantified under a thermal lens microscope after washing out unbound species. Unfortunately, this assay could not be used for in situ analysis nor could it undergo ligand rearrangement to accommodate multivalent receptors.

In the experiments described below, we have designed a multivalent immunoassay for monitoring the binding of bivalent anti-dinitrophenyl (anti-DNP) antibodies to phospholipid bilayer surfaces containing dinitrophenyl haptens. The DNP/anti-DNP system was chosen because it has been well characterized with phospholipid bilayers.[30−35] The assay was fully incorporated into a microfluidic network where a dozen concentration versus coverage data points were obtained simultaneously in a highly accurate, rapid format requiring only minimal amounts of protein analyte. Surface specificity was achieved by using total internal reflection fluorescence microscopy. This technique allowed the surface coverage to be monitored in the presence of high concentrations of bulk analyte.

## EXPERIMENTAL SECTION

All immunoassay experiments were conducted inside linear arrays of bilayer coated microchannels (Figure 1). Each channel was 50 $\mu$m in width and separated from its neighbors by 25-$\mu$m barriers. The device was formed by placing lithographically patterned poly(dimethylsiloxane) (PDMS) molds (Dow Corning Sylgard Silicone Elastomer-184, Krayden, Inc.) into conformal contact with planar borosilicate cover slips.[36,37] The molds were made by curing the low molecular weight polymer with a cross-linker on a photolithographically patterned master prepared by exposing and developing a photoresist patterned surface. The elastomeric mold, which bore the negative pattern of the master, was carefully peeled off and washed repeatedly with acetone and ethanol. Before contact was made with the glass substrate, the PDMS surface was rendered hydrophilic by oxygen plasma treatment for 15 s (plasma cleaner PDC-32G, Harrick Scientific, Ossining, NY). The glass cover slips employed in these experiments were cleaned in hot surfactant solution (ICN ×7 detergent, Costa Mesa, CA), rinsed at least 20 times in purified water from a NANOpure ultrapure water system (Barnstead, Dubuque, IA), and then baked in a kiln at 400 °C for 4 h before use.

Once the hydrophilic microchannels were formed, buffer solutions containing ~1 mg/mL concentrations of small unilamellar vesicles (SUVs) were injected into the channels through 500-$\mu$m holes, which were patterned into each end of each channel as inlet and outlet ports. Supported bilayers formed spontaneously on the channel surfaces via the vesicle fusion method.[10] Excess lipid vesicles were then flushed from the microchannels by flowing pure buffer solution until the channels were judged to be clear of free vesicles by epifluorescence microscopy. All supported membranes primarily consisted of egg phosphatidylcholine (egg PC, Avanti Polar Lipids, Alabaster, AL) doped with a small concentration of phosphatidylethanolamine lipids conjugated with dye groups and haptens covalently attached at the free amine. The dye-labeled lipids, Texas Red DHPE and fluorescein-DHPE, were purchased from Molecular Probes, Eugene, OR. SUVs were formed by standard procedures.[38] Briefly, mixtures of lipids, probes, and haptenylated lipids were mixed in chloroform and added to a clean round-bottom flask. The chloroform was then evaporated under a stream of dry nitrogen followed by further drying for 2 h under vacuum. The dried lipids were reconstituted in the desired buffer solution to a concentration of 1.7 mg/mL. Small unilamellar vesicles were formed by probe sonication of the suspension to clarity followed by centrifugation at 38 000 rpm for 30 min to remove titanium particles. The supernatant was then centrifuged again at 52 000 rpm for 3 h to remove large lipid aggregates. The SUVs were stored at 4 °C before use.

Membranes containing *N*-dinitrophenylaminocaproylphosphatidylethanolamine (DNP-Cap PE, 16:0), which was also purchased from Avanti, were used in the ligand−receptor binding studies. Polyclonal anti-dinitrophenyl-KLH IgG antibody was purchased from Molecular Probes and labeled with Alexa Fluor 594 using a standard antibody labeling kit (A10239, Molecular Probes). Labeling the antibody yielded 2.4 fluorophores/protein as judged by UV absorption spectroscopy. The dye-labeled protein was concentrated to 13.2 $\mu$M by evaporation of water in an evacuated desiccator. The material was stored in a pH 7.2 buffer solution containing 0.01 M sodium phosphate, 0.15 M NaCl, and 0.2 mM sodium azide before use.

Images of the supported membranes and antibodies in the microchannels were obtained by epifluorescence microscopy (E800 fluorescence microscope, Nikon) using a ×10 objective. Total internal reflection experiments were performed to discriminate between antibodies in solution and those bound to the surface.[39] These experiments were conducted by reflecting a 1-mW, 594-nm helium neon laser beam (Uniphase, Manteca, CA) off the sample surface through a dove prism optically coupled to the planar cover slip surface through immersion oil. The laser

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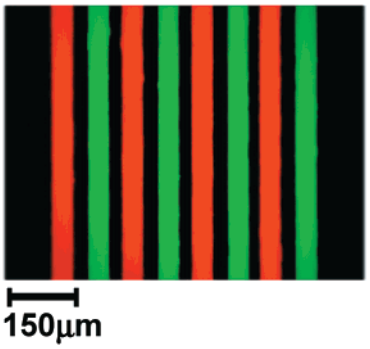


Figure 2. Epifluorescence image of a spatially addressed array of eight egg PC bilayer-coated microchannels. The channels are alternately coated with bilayers containing 1 mol % Texas Red- and 3 mol % fluorescein-labeled lipids.

beam was telescoped out with a ×5 lens set to create an incident intensity profile that varied less than 3% across the width of the microchannel array.

All images were captured with a Photometrics Sensys CCD camera and stored using Metamorph software from Universal Imaging Cor. The images were then transferred to Adobe Photoshop and processed using false color imaging techniques.

## RESULTS

**Preparing Bilayer-Coated Microchannels.** Figure 2 shows the epifluorescence image of an array of eight microchannels coated with fluorescently labeled supported lipid bilayers. Each microchannel was addressed individually by injecting SUVs in a 10 mM PBS buffer at pH 7.2 into the channel inlet ports. Every other microchannel contained 1 mol % Texas Red DHPE probes in the lipid bilayers, while the rest were prepared with 3 mol % fluorescein DHPE probes. Vesicle fusion occurred on both the PDMS channel walls[40] and the underlying borosilicate substrate. Fluorescence recovery after photobleaching experiments indicated that the supported membranes were mobile on both materials. It should be noted that vesicle injection was performed in all channels within 3−4 min after exposing the PDMS mold to the oxygen plasma. This not only ensured that the channels were sufficiently hydrophilic to induce flow by positive capillary action but also led to the formation of high-quality bilayers on the PDMS surface. Uniform formation of the bilayers on the polymer walls as well as the borosilicate substrate was important because it rendered the entire microchannel surface resistant to nonspecific adsorption of IgG antibodies in subsequent immunoassay experiments. Control experiments showed that protein adsorption to bilayer-coated channels was suppressed by at least 2 orders of magnitude in comparison with uncoated channels as judged by quantitative fluorescence measurements. Furthermore, the bilayers appeared to be stable on the microchannels for at least several weeks.

**Designing a Heterogeneous Binding Assay.** To obtain sufficient data for quantitative measurements of ligand−receptor binding as a function of antibody concentration, measurements were made over 2 orders of magnitude in protein concentration. Twelve microchannels were arrayed on a single chip. Each channel was injected with a solution of small unilamellar vesicles

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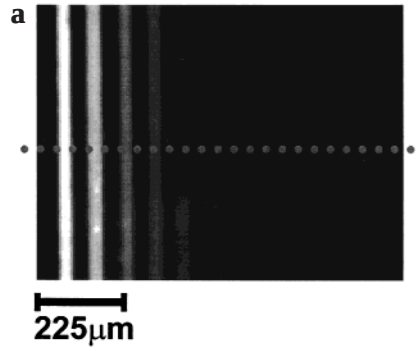


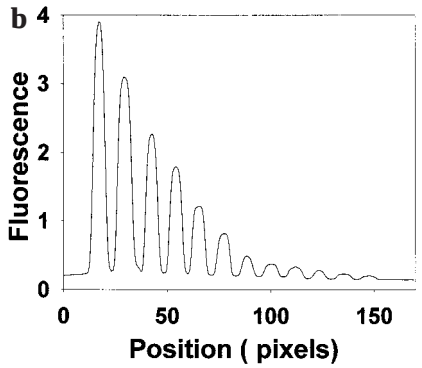


Figure 3. (a) Bulk-phase epifluorescence image of Alexa 594 dye-labeled anti-DNP inside bilayer-coated microchannels. Starting from the left-hand side, the antibody concentrations are 13.2, 8.80, 5.87, 3.91, 2.61, 1.74, 1.16, 0.77, 0.52, 0.34, 0.23, and 0.15 $\mu$M, respectively. A line scan of fluorescence intensity (dotted line) across the microchannels is plotted in (b).

composed of 92 mol % egg PC, 5 mol % DNP-Cap PE, and 3 mol % fluorescein-DHPE in a 10 mM PBS buffer at pH 7.2. After excess vesicles were flushed out, the channels were imaged by epifluorescence microscopy. At this point, various concentrations of Alexa dye-labeled anti-DNP were injected into the channels, with the highest protein concentration on the left side and the lowest on the right (Figure 3a). Because surface binding causes bulk concentration depletion, protein solution flowed continuously through the channels until the bulk concentration became stabile. This required an aliquot roughly equal to 4 or 5 times the channel volume in the microchannels with the lowest protein concentration, while the highest concentration channels required considerably less flow, as expected. A line profile generated from the epifluorescence image of these channels is shown in Figure 3b. The majority of the signal emanated from the bulk.

To obtain information on surface coverage, it was necessary to discriminate between surface-bound antibodies and the vastly larger population in the bulk solution. This was achieved by total internal reflection fluorescence microscopy (TIRFM).[39] TIRFM creates an evanescence wave that decays as a function of distance from the surface as

$$I(z) = I_0 e^{-z/d} \quad \text{and} \quad d = \frac{\lambda_0}{4\pi}[n_1^2 \sin^2 \theta - n_2^2]^{-1/2}$$

where $I_0$ is the intensity of the incident light beam, $z$ is the distance from the interface measured in the normal direction, $n_1$ is the index of refraction of medium 1 (high index medium from which

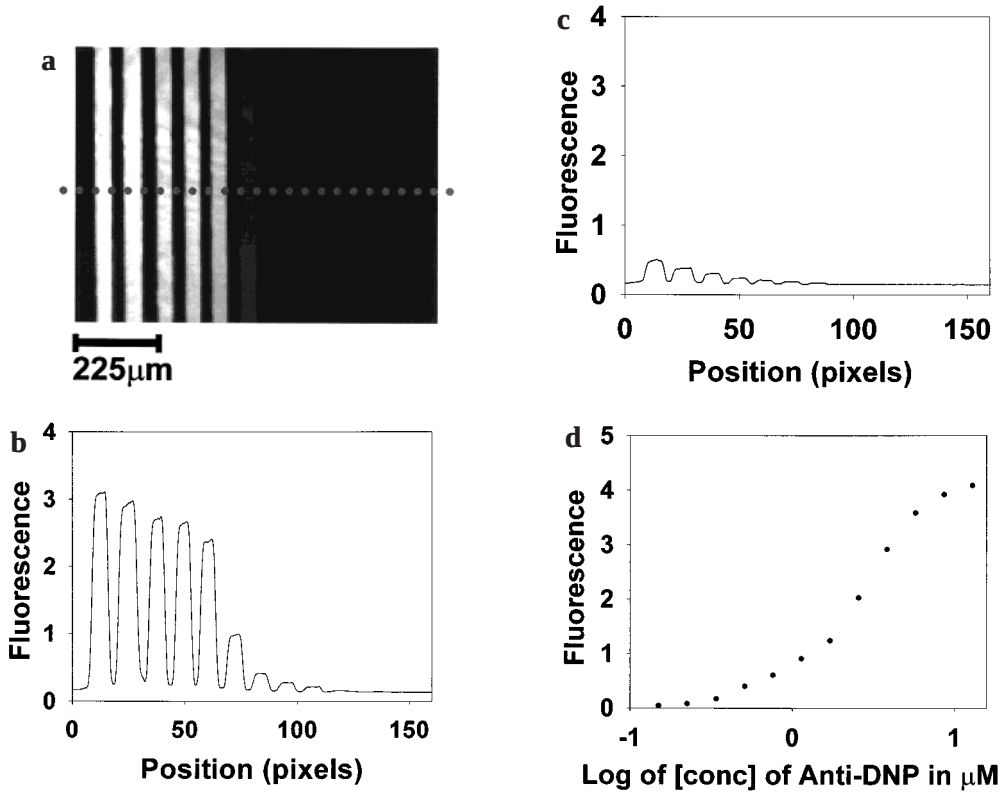


Figure 4. (a) Total internal reflection fluorescence image of the same conditions as shown in Figure 3a. (b) Line scan of fluorescence intensity (dotted line) across the microchannels. (c) Line scan of fluorescence intensity across bilayer-coated microchannels similar to the membranes in (a) but without any DNP-labeled lipids. (d) Point-by-point plot of the intensity in each channel in (b) after subtracting the background intensity of (c).

the light is incident), $n_2$ is the index of refraction of medium 2 (low index medium into which the evanescent field is propagating), $\theta$ is the angle that the radiation makes with the surface normal and, $\lambda_0$ is the wavelength. The substrate beneath the channels on which the bilayers resided was made of borosilicate float glass, which has an index of refraction of ~1.52. Inside the channels above this surface was the aqueous phase with an index of refraction close to 1.33. Since the laser beam was incident on this surface at ~79°, the intensity of the evanescence field fell to 37% (1/e) of its initial value at a distance of 70 nm above the liquid/solid interface for the 594-nm radiation employed.

Figure 4a shows the same system as (3a), but now imaged by TIRFM. As can be seen from the line profile (Figure 4b), the fluorescence intensity was no longer linear with respect to bulk concentration. The signal, however, represented a combination of specifically bound antibody, nonspecifically bound antibody, and near-surface antibody in the bulk solution. To account for signal arising from the latter two effects, the experiment was repeated under the identical conditions, but in the absence of DNP-Cap PE lipids in the SLBs. In this case, the fluorescence intensity from the total internal reflection experiments was dramatically weaker as demonstrated by the line profile (Figure 4c). After flowing the antibody out of the channels, the TIRFM image was repeated and the signal fell by more than 1 order of magnitude with respect to Figure 4c (data not shown). This last experiment demonstrated that most of the background signal arose from either near-surface bulk proteins or rather weakly bound surface species rather than from irreversibly adsorbed IgG. A binding curve for coverage versus bulk protein concentration was obtained for the DNP/anti-DNP system by subtracting the background in Figure 4c from the data in Figure 4b. This is plotted on a semilog scale in Figure 4d. The half coverage point on the curve occurs at a concentration of 1.8 $\mu$M and is in good agreement with data from previous experiments.[31,41]

## DISCUSSION AND CONCLUSIONS

An important feature of the SLB/microfluidic techniques developed here is that all data points are collected simultaneously. In addition to merely being more rapid than assays that obtain data sequentially, the measurements should also be more accurate, as sources of error such as changes in flash lamp intensity over successive measurements are eliminated. These methods also use a smaller quantity of protein than previous techniques; however, measurements of binding curves in heterogeneous assays require more analyte than sensors designed merely to detect the presence of such species. The volume of protein solution used to fill the microchannels in these experiments was ~2 $\mu$L when the injection port volume is included. Furthermore, it was necessary to flow excess protein solution through each channel to account for bulk depletion by surface adsorption. In total, ~35 $\mu$g (230 pmol) of IgG antibody was used to perform a single multivalent binding constant measurement. It should be possible to reduce this amount by 1 order of magnitude or more by using smaller channels, syringes, and injection ports.

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