Evaluation of a Three-Dimensional Micromixer in a Surface-Based Biosensor†

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The performance of many biosensors is limited by the rate at which a soluble analyte solution is transported to a complementary receptor immobilized on the sensor transducer. In these cases, transport limitations due to the lack of sample mixing in the sensor can impede analyte transport to the immobilized receptors and thereby reduce the sensor performance. In this study, we apply methods that have been successfully used to mix bulk fluid streams in a microfluidic device, to stir the analyte solution as it passes over receptors bound to the surface of an optical biosensor. A three-dimensional serpentine microchannel, which has been shown to provide chaotic mixing in the sample flow, was interfaced with a surface plasmon resonance biosensor. The binding kinetics of soluble rabbit IgG to protein A, immobilized on one of the microchannel walls, was measured both in the serpentine device and in a straight channel. A comparison of the initial rates of analyte detection in these two devices shows that the mixing characteristics in the serpentine microchannel can double the initial rate of analyte detection. The serpentine mixer is thus a promising method for improving the performance of surface-based microfluidic biosensors.

Introduction

In many biosensor devices, detection occurs when a soluble analyte binds with a complementary, surface-immobilized biomolecule. When this recognition process is diffusion-limited, the sensor's response time and practical detection limits can be determined by analyte mass transport. One way to accelerate mass transport and improve sensor performance is to stir the analyte as it passes through the device. Unfortunately, stirring or mixing fluids in current biosensors is not straightforward. To maximize portability and minimize reagent consumption, a large number of biosensors are now constructed on a microfluidic platform.[1,2] The small scale of these systems precludes the use of turbulent flow. Moreover, the relatively large surface area to volume ratios of these devices makes mechanical stirring difficult.

A substantial effort has focused on devising mixing strategies that circumvent these problems.[3−5] So far, researchers have developed methods for mixing two fluid streams in a microchannel. The general approach has been to increase the flux of material between the two streams by decreasing the length and increasing the contact area over which diffusion occurs. For example, Branebjerg et al. did this by splitting two input streams with a cascade of separation plates into multiple streams and then recombining them into several thin, closely spaced layers.[3] Several other mixing schemes adopt the same approach but use oscillatory pressure gradients, electric fields, or traveling ultrasonic waves to stretch, elongate, and fold the interface between the two fluids.[6−8] In these devices, this fluid deformation manipulates the contact area and the diffusion length in order to mix the sample.

Another approach has been to use "chaotic advection".[9,10] With this method, laminar flows that exhibit seemingly random and chaotic particle trajectories are used to mix multiple fluid streams. Relative to normal laminar flow, in which fluid particles largely move in a single direction, these chaotic flows exhibit more three-dimensional particle motion.[11] This added dimensionality stretches and folds

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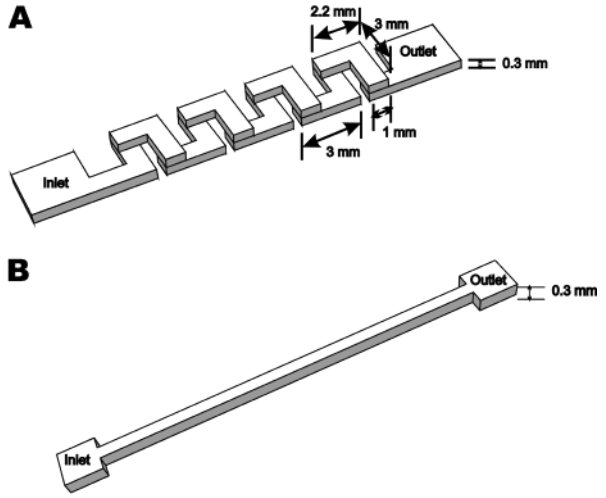

Figure 1. The designs of the three-dimensional serpentine (a) and straight (b) microchannels. These illustrations are not drawn to scale.

fluid−fluid boundaries, again manipulating the diffusion length and contact area to enhance mixing.

As mentioned, the above strategies only address mixing between bulk solutions. While useful for solution bioassays, this is not particularly relevant to surface-based biosensors. In these devices, one must stir a single fluid stream (i.e., mix analyte-rich and analyte-poor sections of the sample) as it passes above a reactive surface. To our knowledge, no studies have investigated the effects of bulk sample mixing or stirring in such microfluidic sensors. All of the above mixing strategies accelerate mass transport by simultaneously increasing the interfacial area and decreasing the diffusion length between multiple fluid streams. Since these features can also enhance analyte transport within a single sample stream, it should be possible to effectively apply strategies for mixing bulk fluid streams to enhance transport to the channel walls in surface-based biosensors.

In this study, we investigated this possibility by using "chaotic advection", to stir a sample solution as it flows through a microfluidic biosensor device. In particular, we studied the performance of a microscale, three-dimensional serpentine micromixer (Figure 1a) in a surface plasmon resonance (SPR) biosensor. Previous numerical simulations indicate that flow in this geometry can exhibit "chaotic advection".[11] Liu et al. and Stremler et al. experimentally tested these numerical results by studying the mixing and reaction of separate phenolphthalein and sodium hydroxide streams in this and other microchannel geometries.[5,11] Their results clearly demonstrated that the three-dimensional serpentine channel improves mixing performance; namely, the three-dimensional channels produced 16 times more reacted phenolphthalein than a simple straight channel. Moreover, by visualizing the distribution of phenolphthalein in several channel designs, they showed that a three-dimensional serpentine design produces a more uniform and well-mixed distribution of reacted phenolphthalein than alternative geometries of the microchannels. One would deduce, therefore, that enhanced mixing between fluid streams in the latter system would similarly enhance analyte transport to detector molecules immobilized on the walls of a biosensing device.

To test this, we measured the kinetics for the binding of IgG to immobilized protein A both in the above serpentine microchannel and in a simple straight channel of equivalent length. In these experiments, soluble rabbit IgG antibodies were passed through each microchannel and allowed to bind to protein A, which was immobilized on one of the microchannel walls. The importance of analyte mass transport in this biorecognition system can be determined from the nondimensional Damköler number.

$$\kappa = k_f c_{b,sat} h/D \quad (1)$$

The above expression is the ratio of the rate of IgG diffusion to the rate of IgG−SpA binding, where k_f is the kinetic on rate for IgG−SpA binding reported in M^{-1} s^{-1}, c_b is the surface density of SpA immobilized on the microchannel wall, h is the microchannel depth, and D is the diffusion constant for the soluble IgG. Estimates of the Damköler number based on published kinetic data, along with previous measurements of antibody diffusivity and SpA surface densities, suggest that the diffusion of IgG is 10−100 times slower than the intrinsic kinetics of IgG−SpA binding.[12,13] This biorecognition system should therefore be diffusion-limited and a function of the extent of mixing above the sensor surface. While the serpentine configuration can provide mixing via "chaotic advection", there should be little or no mixing in the straight channel since the convective flux in this device is parallel to rather than toward the sensor walls.[14] A comparison of the detection kinetics in these two geometries shows that serpentine micromixers may be useful tools not only for enhancing mixing in the bulk but also for enhancing the performance of microfluidic biosensors.

Materials and Methods

Reagents. Recombinant Protein A (rSpA) was purchased from Calbiochem (San Diego, CA). The analyte, polyclonal rabbit IgG, was purchased from Sigma-Aldrich Corp. (St Louis, MO) along with *N*-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine, and bovine serum albumin (BSA). The following materials were used for microchannel fabrication: poly(dimethylsiloxane) (PDMS) (SYLGARD 184 elastomer kit, Dow Corning, Midland, MI), XP SU-8 100 negative UV photoresist (Microchem Corp., Newton, MA), and 3 in. diameter polished silicon wafers (Montco Silicon Technologies, Royersford, PA). Glycine-HCl, acetic acid, NaN_3, and Tween-20 (T-20) were purchased from Fisher Scientific (Pittsburgh, PA).

Microchannel Design. The design of the serpentine microchannel appears in Figure 1a. This design is similar to previously described devices.[5,11,15] It consists of four mixing segments placed in series. Each mixing segment is formed by stacking two, in-plane, L-shaped channel sections on top of one another. Although adjacent sections have slightly different dimensions, the orientation of each L-shaped segment is a mirror image of its adjoining neighbor. Each mixing segment guides the sample through an L-shaped section, rotates the fluid by 90°, and then passes the flow through an adjoining L-shaped section. When several mixing segments are linked together, the flow is subjected to a series of bends and turns that twist the fluid through a series of orthogonal planes.

Figure 1b illustrates the straight channel design. The height and width of the channel are identical to the corresponding dimensions in the serpentine mixer (Figure 1a). In addition, the total length of the channel was adjusted so that the sample residence time is the same in both devices.

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Microchannel Fabrication. The microchannels were patterned into thin layers of PDMS using previously described micromolding and replica molding techniques.[15,16] Briefly, the layout of the microchannel was printed on transparency film with a high-resolution laser printer. Transparencies were subsequently used as masks in contact photolithography to transfer the channel design onto silicon wafers spin-coated with a negative UV photoresist. Master templates for replica molding were finally formed by developing the photoresist to generate a positive relief mold of the channel design on the silicon wafer.

A sandwich, replica-molding procedure was used to construct microchannels in thin layers of PDMS. A PDMS prepolymer mixture was prepared by mixing the contents of the SYLGARD 184 elastomer kit in a 1:10 weight ratio for 10−15 min. This mixture was then degassed and poured onto the master. To form a thin PDMS membrane, the master with the prepolymer mixture was clamped between two flat plates and cured on a hot plate for 8 h at 100 °C. Afterward, the clamp assembly was disassembled and the cured PDMS membrane, which then contained a pattern of the microchannel, was peeled off the master.

The above procedure is only useful for creating the straight channel and other two-dimensional, planar designs. The three-dimensional channel geometry of this device was constructed by patterning its features into two thin layers of PDMS and then stacking these layers on top of one another.[15] One layer contained the L-shaped sections that form the bottom half of the mixer while the other contained the complementary L-shaped regions that form the upper half of the device. The PDMS layers were constructed as described above and then bonded together. Bonding was accomplished by activating each layer in an oxygen plasma (70 W, 75 mTorr) in a rapid ion etching (RIE) system for 10−15 s. The PDMS samples were then rinsed with methanol and brought into contact. After the two layers were aligned by hand under a stereomicroscope, the PDMS assembly was placed on a hot plate at 85 °C in order to evaporate any remaining methanol. As the sample dried, an irreversible seal spontaneously formed between the two layers.[15]

In many cases, the surfaces of the PDMS microchannels were hydrophobic after fabrication. They are not easily wetted by aqueous solutions, are prone to nonspecific protein adsorption, and can easily nucleate air bubbles. These features could lead to microchannel clogging and/or complicate the binding kinetics. To avoid these potential difficulties, all devices were retreated with an oxygen plasma after stacking and bonding the PDMS layers as described above. All PDMS devices were again exposed to an oxygen plasma (70 W, 75 mTorr) for 10−15 s. These conditions were sufficient to render the microchannel walls hydrophilic.[17] Unless stored in an aqueous environment, these surfaces will regain their hydrophobic character.[16,18] To prevent this from happening, all devices were stored in a blocking buffer containing 2 mg/mL BSA in PBS (phosphate-buffered saline, pH 7.4) immediately after removal from the plasma chamber and until use.

Array SPR Biosensor. The array SPR biosensor illustrated in Figure 2 was used to monitor antibody binding. The instrument contains a Teflon fluid cell with connection ports for external tubing. The patterned PDMS membranes described above are sandwiched between a protein-coated sample surface and the Teflon cell. This assembly is then clamped together and placed in optical contact with an equilateral BK-7 prism (Edmund Scientific, Burlington, NJ). A small range of incident angles is sampled by using a lens system to focus p-polarized, collimated light from a diode laser (Meredith Instruments, Glendale, AZ) onto the sample. The reflected light is collected with a TSL1401 photodiode array (Texas Instruments, Dallas, TX) that contains 128 linearly arranged pixels. Resonance curves were measured by plotting each pixel's signal against its position in the array. A custom-written computer program then measured complete resonance curves at user-specified time intervals. Kinetic data were collected by recording the shifts in the minima of the resonance curves as a function of time. The magnitudes of these shifts, and therefore the change in the protein surface density, were quantified in terms of the number of pixels the resonance angle had shifted from an established baseline signal.

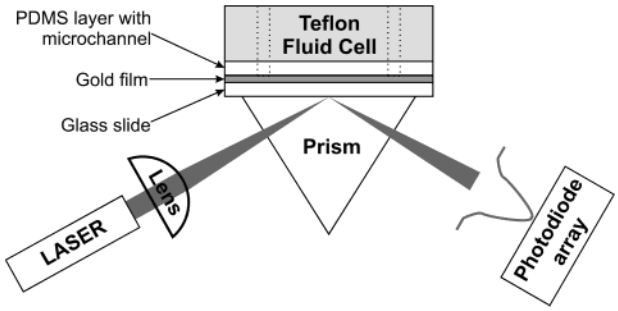

Figure 2. A schematic illustration of the array SPR instrument.

Immobilization of Recombinant Protein A. Recombinant protein A was covalently immobilized on gold-coated glass substrates. To minimize sample-to-sample variation in the immobilized protein density, all samples were prepared in a single batch. Ten microscope slides (Corning, Corning, NY), measuring 2.5 × 7.5 cm, were cleaned in a warm solution of deionized water, HCl, and H_2O_2 (1:1:1). The slides were then rinsed with water and sonicated for 30 min. After being rinsed again, the slides were dried under a stream of filtered nitrogen. A 10 Å thick chromium adhesion layer was deposited on one side of the microscope slides via thermal evaporation (Cooke Vacuum Products, Norwalk, CT). A 500 Å gold film was then evaporated onto the chromium. After being allowed to cool for 10−15 min, the slides were removed from the evaporator and immersed in a 2 mM solution of 11-mercaptoundecanoic acid in ethanol for 12 h at room temperature. The substrates were then removed and rinsed thoroughly with absolute ethanol.

Protein A was then coupled to the alkanethiol surface via its primary amine groups. The microscope slides, coated with the above gold and organothiol films, were placed in a large Petri dish and incubated in a solution of 3.8 mg/mL EDC and 6.7 mg/mL NHS for 15 min at room temperature. This procedure activates the carboxylic acid groups with the amine-reactive NHS moiety. After the slides were rinsed with water, protein A was coupled to the NHS-activated surface by incubating the microscope slides for 4 h in a 0.05 mg/mL solution of rSpA in PBS. Following the incubation, the slides were treated with a 1 M ethanolamine solution at pH 8.5 for 15 min to quench the amine coupling reaction, rinsed with water, and stored in PBS until use.

Kinetic Assays. The kinetics for the binding of soluble antibody to immobilized protein A were measured for both the straight and serpentine microchannels. To do this, a PDMS membrane, which contained a pattern of the desired microchannel, and an rSpA-coated microscope slide were clamped to the SPR fluid cell. The assembled cell was then connected via Teflon tubing to a syringe pump. Before the binding kinetics were measured, PBS buffer with 0.1% T-20 was injected in the fluid above the sample surface, and a baseline signal was recorded. A 1 μg/mL solution of rabbit IgG in PBS was then pumped through the fluid cell at a specified flow rate. After the flow was initiated, data were recorded every 30−60 s until the signal equilibrated. The binding time course was next measured at three different flow rates: 2, 10, and 40 mL/h. All flow rates were tested with a single sample, and multiple kinetic measurements were conducted in each experiment. Between assays, the immobilized protein A was regenerated by passing a mixture of 1 M acetic acid and 0.1 M glycine−HCl at pH 3.3 through the SPR fluid cell for 5 min. Before the next assay was begun, the sample was equilibrated with PBS at pH 7.4 for 5−10 min.

Measuring the Surface Density of Immobilized Protein A. To ensure that the protein A activity was the same in all of the samples, the shift in the resonance curve due to protein A immobilization, and therefore the rSpA surface density, was measured on four different substrates. All substrates were

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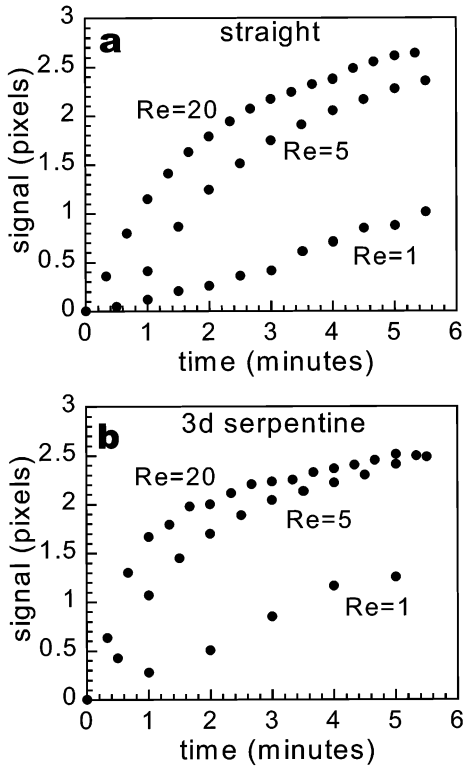

Figure 3. The IgG/SpA binding kinetics measured in the (a) straight and (b) three-dimensional serpentine microchannels.

modified in a single batch as described above. Each sample was mounted in the SPR fluid cell. After the samples were rinsed with PBS, five to six resonance curves were recorded. The fluid cell was then disassembled, and the samples were placed under a UV mercury lamp for 8 h. The UV radiation removed the protein by oxidizing all of the organics on the gold substrate. Following the UV treatment, samples were rinsed with ethanol, dried under a stream of nitrogen, and returned to the SPR fluid cell. Resonance curves were measured again, and compared with the corresponding data recorded at the beginning of the experiment. The resulting shift in the resonance curves was used as a measure of the density of immobilized rSpA.

Results

Flow Rate Analysis in the Straight Microchannel. In order for mixing to improve biosensor performance, the binding, and hence the detection kinetics, must be diffusion-limited. The calculated Damköler numbers for this system ranged from $\kappa = 10$ to 100, which suggests that the IgG/protein A association is diffusion-controlled. However, since this calculation is based on estimates for the kinetic rate constants and the rSpA surface density, it is only a rough indicator of mass transport limitations in our experiments. To experimentally confirm the relevance of mass transport in this study, we measured the rSpA/IgG association kinetics in the straight channel as a function of the assay buffer flow rate. Figure 3a shows the binding dynamics at three different flow rates. The flow rates are presented in terms of the corresponding nondimensional Reynolds numbers

$$\mathrm{Re} = \rho Q/\mu w \qquad (2)$$

where ρ is the density of the assay buffer, Q is the volumetric flow rate, w is the width of the microchannel, and μ is the viscosity of the buffer. The apparent analyte binding kinetics scale with the Reynolds number—that is, with the fluid velocity (Figure 3a). The binding dynamics must therefore be a function of the analyte transport within the microchannel, confirming that the kinetic assays performed in this study are diffusion-limited.

Table 1. A Comparison of the Initial Rates for IgG/SpA Binding in the Straight and Serpentine Channels[a]

Reynolds no.	straight channel (pixels/min)	serpentine channel (pixels/min)
1	0.12	0.25
5	0.62	1.1
20	1.2	1.9

[a] All values were calculated from the data in Figure 3.

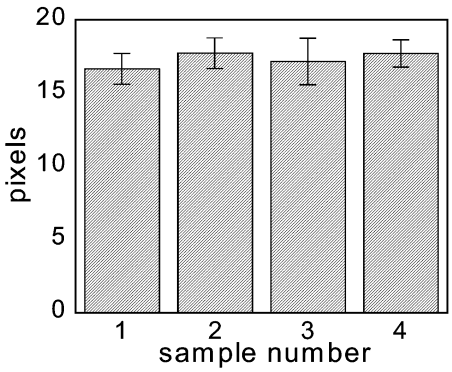

Figure 4. The shift in the resonance angle after immobilization of protein A on four different sample surfaces. The shift in the resonance in the angle is directly proportional to the density of surface-bound protein A.

Flow Rate Analysis in the Serpentine Microchannel. The above determination of the binding rate dependence on the fluid velocity was repeated in the three-dimensional serpentine microchannel. In this design, IgG/rSpA binding was again diffusion-limited (Figure 3b). To compare the detection kinetics in the straight and serpentine channels, the initial binding rates were calculated for the data in Figure 3. These values were calculated from the slope of the first three points in each binding time course. For all of the tested Reynolds numbers, the initial rates increased by a factor of 2 in the serpentine channel relative to those in the straight channel. Therefore, although the serpentine design did not eliminate transport limitations, these data indicate that it did enhance the mixing, and thereby accelerated the binding rates.

Evaluation of SpA Surface Activity. To accurately compare the performance of the straight and serpentine channels, the activity of the immobilized protein A must be the same in all experiments. If the binding capacity is not constant, it is impossible to determine whether differences in the observed sensor performance are due to the choice of channel design and flow rate or simply due to variations in the receptor activity. The latter possibility could arise from (1) the gradual denaturation of the immobilized SpA due to repeated sample regeneration or (2) sample-to-sample variation in the surface density of immobilized protein A. Several control experiments were performed to test for these potential sources of variability. Figure 4 illustrates the sample-to-sample variation in our experiments. Here, the variability in the surface density of protein A was measured by preparing four samples and then measuring the surface density of rSpA on each of them, with SPR. There was no observable sample-to-sample variation since all of the signals in Figure 4 are identical within experimental error. The effect of sample regeneration was also investigated. Figure 5 shows the equilibrium signals from 10 consecutive kinetic assays. Between assays, the immobilized protein A was regener-

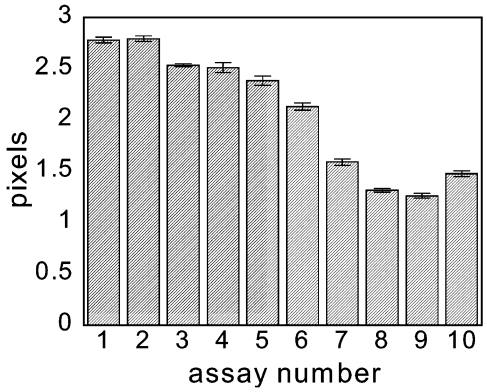

Figure 5. The effect of the acid regeneration protocol used between kinetic assays. The equilibrium signal was recorded over 10 consecutive kinetic assays. Between each assay, the sample surface was regenerated with 1 M acetic acid, 0.1 M glycine−HCl, and pH 3.3 solution.

ated with a low pH buffer as described. These data show that the protein A activity was constant for the first three to four assays but gradually decreased in subsequent measurements. To ensure that the low pH regeneration protocol had a minimal effect on the results, all of the data in Figure 3 were collected from the first three kinetic assays measured with each sample.

Discussion

The three-dimensional serpentine microchannel clearly improves the performance of the SPR biosensor. It offers faster IgG/rSpA binding kinetics and thus must also provide more extensive stirring and faster antibody transport than the straight channel. Despite this, from a practical perspective, the serpentine channel only offers a moderate enhancement in performance over its straight counterpart. In particular, the serpentine's faster detection kinetics are not so impressive given that much larger rates (an order of magnitude or more) are usually needed to justify the additional cost of fabricating the serpentine's more complex three-dimensional geometry.

The serpentine geometry only offers a modest improvement in performance because it does not eliminate mass transport limitations from the IgG/rSpA binding kinetics. The rate of binding along the bottom of the channel is still faster than the rate of antibody transport. Although the faster kinetics in Figure 3b indicate that these rates become more comparable, the mass flux of antibody toward the sensor surface must be increased further to realize additional improvements in sensor performance.

One can further enhance analyte transport by altering the channel design. For example, the frequency of the L-shaped sections in the serpentine's design could be increased. By making this change, the fluid would experience more bends and turns that would tend to simultaneously decrease the diffusion length and increase the contact area between analyte rich and analyte poor sections of fluid. Ultimately, this additional distortion of fluid−fluid boundaries within the sample solution would enhance analyte transport.

Modifying the channel design will affect transport rates in the bulk sample solution. It is also possible that diffusion limitations are a partial or complete consequence of an unstirred layer near the sensor surface. In the case where the sample flow undergoes turbulent mixing, boundary layer theory indicates that there is a viscous, unstirred layer near the channel walls.[14] Since turbulence is one example of a chaotic flow, this phenomenon may extend to the sample flows in this study. If this is the case, despite extensive mixing via "chaotic advection" in the bulk solution away from the channel walls, an unstirred layer may form a concentration boundary layer near the sensor surface that will limit the rate of analyte detection.

In summary, we compared the mixing capabilities of a straight and serpentine microchannel in a surface-based biosensor. The detection of soluble rabbit IgG by immobilized protein A was approximately two times faster in the serpentine channel. Given the added effort and cost needed to construct the serpentine channel, this only represents a slight improvement over the simpler straight channel. Nevertheless, these results are promising. The three-dimensional serpentine geometry did enhance the apparent sensor performance. By altering the serpentine channel's design, it may be possible to obtain even better sensor performance.

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