Anal. Chem. **2001,** *73,* 5896−5902

Flow-Based Microimmunoassay

Mark A. Hayes,*,† Nolan A. Polson,‡ Allison N. Phayre,† and Antonio A. Garcia§

Department of Chemistry and Biochemistry and The Center for Solid State Electronics Research, Arizona State University, Tempe, Arizona 85287, ThermoBiostar, Inc., Boulder, Colorado 80301, and Department of Bioengineering, Arizona State University, Tempe, Arizona 85287

A small-volume heterogeneous immunoassay system is demonstrated in microchannels exploiting magnetic manipulations of small paramagnetic particles (1−2-μm diameter). The small-diameter particles help to create a high surface-area-to-volume ratio that generates the sensitivity for the small detection volumes. Flow characteristics of the magnetically formed packed bed within the channel allow the assay to be carried out quickly (minutes) while passing appropriate volumes of both samples and reagents (microliters to hundreds of nanoliters) through the system. The assay is demonstrated with a direct interaction of fluorescein isothiocyanate (FITC) with an immobilized anti-FITC conjugate in which a small-volume sample (<1 μL) reaches 90% of maximum signal in 3 min. Heterogeneous sandwich assays are demonstrated with parathyroid hormone (PTH) and interleukin-5 (IL-5). Both the PTH assay and IL-5 assays were carried out on microliter volumes and demonstrated physiologically relevant sensitivity (~μg/L).

The immunoassay is arguably the most important analysis method for biological molecules. The molecular recognition interaction provides high sensitivity and chemical selectivity; features that are especially important when considering the measurement of specific biological molecules in the milieu of similar structures present in typical samples. Immunoassays are a popular method in many clinical applications and form the basis of a wide array of biochemical, environmental, and biological investigations.[1−3]

Although the conventional microtiter plate assays will continue to be a valuable paradigm, there are a number of applications that can benefit from alternative approaches. Some of these approaches are flow injection analysis methods, small-volume separation-based methods, and microfluidic devices.[4−8] The benefits of these alternative approaches include compatibility with flow-based systems, small reagent consumption and waste generation, faster overall analysis time, convenient or localized detection, increased temporal information content, and simplified automation. In the flow injection format, surface plasmon resonance has served as a direct detection scheme for unlabeled antibody−antigen interaction for heterogeneous assays.[4] The detection is based on changes in the refractive index of carefully prepared surface layers within relatively expensive instrumentation. The limit of refractive-index-based detection is that small molecules with buffer-like properties or properties similar to the surface layers are difficult to detect. On microdevices, enzyme assays, DNA binding, and competitive immunoassays have been performed in which the direct quantitation of analyte[6−8] and determination of binding/kinetic constants[5,9] have been demonstrated. These experiments were, in contrast with conventional heterogeneous systems, homogeneous immunoassays in which both the antigen and antibody molecules are introduced in solution, complexation takes place, and an electrophoretic separation and detection follows. This is a valuable format, and the separation step can be information-rich and efficient, but it limits the assay to only those systems with significantly different electrophoretic migration rates between complexed and free-form antigens or antibodies. Low-molecular-weight antigens are ill-suited for this format because of the similar electrophoretic migration rates of the free versus the complexed antibody. In a commercial capillary electrophoresis instrument, labeled paramagnetic particles were used with an orthogonal magnetic field to localize the particles.[10] This work demonstrated a separations-based detection system within the small volumes of conventional fused-silica capillaries (75-μm i.d., 2−3-mm packed particle bed). The use of a solid-phase immobilization for small-volume immunoassay allows for a localized assay.[11] This heterogeneous assay system demonstrates many of the advantages of the alternative approaches, such as reduced time and reagent consumption for captured particles within small volumes; however, in this instance, its potential is limited by the solid support size and material and the sequestration mechanism for the bed.

* To whom correspondence should be addressed. E-mail: mhayes@asu.edu.
† Department of Chemistry and Biochemistry and The Center for Solid State Electronics Research, Arizona State University.
‡ ThermoBiostar, Inc..
§ Department of Bioengineering, Arizona State University.

(1) Van Regenmortel, M. H. V. In *Principles and Practice of Immunoassay*; Price, C. P., Newman, D. J., Eds.; Stockton Press: New York, 1997; pp 14−34.
(2) Chan, D. W.; Sokoll, L. J. *J. Clin. Lig. Assay* **1999**, *22*, 3−6.
(3) Hage, D. S. *Anal. Chem.* **1999**, *71*, 294−304.
(4) Sjolander, S.; Urbaniczky, C. *Anal. Chem.* **1991**, *63*, 2338−2345.
(5) Cohen, C. B.; Chin-Dixon, E.; Jeong, S.; Nikiforov, T. T. *Anal. Biochem.* **1999**, *273*, 89−97.
(6) Schmalzing, D.; Koutny, L. B.; Taylor, T. A.; Nashabeh, W.; Fuchs, M. *J. Chromatogr. B* **1997**, *697*, 175−180.
(7) Koutny, L. B.; Schmalzing, D.; Taylor, T. A.; Fuchs, M. *Anal. Chem.* **1996**, *68*, 18−22.
(8) Chiem, N.; Harrison, D. J. *Anal. Chem.* **1997**, *69*, 373−378.
(9) Hadd, A. G.; Raymond, D. E.; Halliwell, J. W.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **1997**, *69*, 3407−3412.
(10) Rashkovetsky, L. G.; Lyubarskaya, Y. V.; Foret, F.; Hughes, D. E.; Karger, B. L. *J. Chromatogr. A.* **1997**, *781*, 197−204.
(11) Sato, K.; Tokeshi, M.; Odake, T.; Kimura, H.; Ooi, T.; Nakao, M.; Kitamori, T. *Anal. Chem.* **2000**, *72*, 1144−1147.

10.1021/ac0104680 CCC: $20.00 © 2001 American Chemical Society
Published on Web 11/10/2001

Beyond these small-volume assays and to fully realize the potential of heterogeneous microimmunoassay in a flow-based system, several design features need to be addressed. The substrate for the immobilized agent should provide a high surface-area-to-volume ratio (S/V) to, first, reduce diffusion distances (and time) for all steps in the procedure and, second, increase the density of binding sites within a given detection volume. To further increase that density, the binding sites per unit surface area should also be maximized, except for competitive assays.[12] The number of binding sites within the detection volume directly impacts the sensitivity of the assay and can influence the overall detection limit.[12] In addition, for ease of use and flexibility, the design should allow for the rapid regeneration or exchange of the solid support as needed. This can be done by either microdevice structures (weir designs, filters, membranes, etc.) or by the use of paramagnetic particles and an orthogonal magnetic field.[10,11,13] The volume of the substrate, typically a packed bed of some type, should not be so small as to allow saturation of the binding sites, nor so large that significant differential binding density occurs over the length of the bed. The volume flow rate must be sufficient to allow the required sample volumes to interact with the bed surfaces. For systems in which the bed occludes the entire flow path, there are significant fluid dynamic limits on volume flow rates with small particle sizes. This may restrict the total bed length, the smallest usable particle size, or the volume flow rate. In optimizing substrate bed volume, the flow rate and volume, and the timing of each interaction, the overall system should be driven to the kinetic limit of the immuno-interaction, which is the ultimate temporal limiting factor.

In this study, a heterogeneous immunoassay is performed in microchannels to demonstrate the advantages of using small particles (1−2-μm diameter), small-volume samples, and paramagnetic materials. In a demonstration immunointeraction, a fluorescein isothiocyanate (FITC)/anti-FITC complexation is shown. The optimized interaction time for this substrate is ∼3 min for a sample volume of a few microliters. This fast interaction time is a result of both the small volumes and the high surface area afforded by the small particles. For more realistic samples, the system is initially demonstrated with a sandwich assay from human plasma for parathyroid hormone (PTH, 1.4 μg/L) with biotinylated anti-parathyroid hormone (anti-PTH) and fluorescently labeled anti-PTH* complex. Finally, a calibration curve is generated at clinically relevant levels (2−250 μg/L) with interleukin-5 (IL-5), an important cytokine for asthma, immune response cascades, and cancer.

EXPERIMENTAL SECTION

Reagents. Sodium dihydrogen phosphate (NaH_2PO_4) and FITC were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI) and were used as received. Biotinylated monoclonal anti-FITC (1 mg/mL); glutaraldehyde, Tween-20, bovine serum albumin (BSA), and thiourea were obtained from Sigma Chemical Company (St. Louis, MO) and used as received. Potassium dihydrogen phosphate (KH_2PO_4) and potassium chloride (KCl) were obtained from EM Science (Gibbstown, NJ) and were used as received. Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), and sodium chloride (NaCl) were obtained from Mallinckrodt, Inc. (Phillipsburg, NJ) and, as well, were used as received. All of the NaH_2PO_4 buffers were prepared to a 20 mM concentration and adjusted to the appropriate pH using 1 M sodium hydroxide (Mallinckrodt, Phillipsburg, NJ). Polybead amino paramagnetic particles (1−2 μm diameter) were purchased from Polysciences, Inc. (Warrington, PA). All buffers and samples were degassed under vacuum for 5 min and were filtered using a Millex-LCR filter unit, 0.5-μm pore size (Bedford, MA) or a Whatman 0.45-μm pore size hydrophilic syringe filter (Ann Arbor, MI). All buffers and samples were prepared using 18 MΩ purified water drawn from a NANOpure UV ultrapure water filtration system (Barnstead, Dubuque, IA).

PTH Assay. Parathyroid hormone standards (1.4 μg/L and controls), biotinylated goat anti-PTH capture antibody (0.260 mg/mL), fluorescein-labeled goat anti-PTH tag antibody (1.0 mg/mL), phosphate-buffered saline (PBS) (0.1 M; 0.4% BSA), and Dynal streptavidin-labeled paramagnetic particles (2.8-μm diameter, 1 mg/mL diluted 5× in phosphate-buffered saline with 0.45% BSA) were gifts from Nichols Institute Diagnostics (San Juan Capistrano, CA).

IL-5 Assay. Biotinylated rat monoclonal anti-mouse IL-5 (0.94 μg/mL) and mouse IL-5 standards (1 mg) were obtained from BD PharMingen (Franklin Lakes, NJ) and were used as received. FITC conjugated rat monoclonal anti-mouse IL-5 (FITC Ab, 0.2 mg/mL) was obtained from either BD PharMingen or Caltag Laboratories, Inc. (Burlingame, CA) and was also used as received. PBS (pH 7.4) with 0.05% Tween-20 (PBS/Tween) was prepared by adding 50 μL of Tween-20 to 100 mL of PBS. A blocking buffer of PBS/1% BSA was prepared by adding 1 g of BSA to 100 mL of PBS.

Procedures. *Preparation of 1−2 μm Particles for Immunoassay.* The Polybead amino-activated microparticles (2.5 wt % particles in water) were washed with deionized (DI) water by adding DI water to the particle suspension and shaking them for 10 min using a dual-action shaker. Isolation of the particles using a rare earth magnet (Dynal Corp, CA) permitted the removal of the supernatant waste. This was repeated three times or until the supernatant was clear. Then, enough DI water was added to reconstitute the original suspension concentration of 2.5 wt % particles. Glutaraldehyde is mildly polymerized at 70 °C based on a previously reported procedure,[14] and 100-fold excess polymer was added to the cleaned particles. This excess was obtained by adding 1 part of the 2.5 wt % particles in DI water and 1 part polyglutaraldehyde. The resulting solution was then placed in a water bath kept at 70 °C and shaken by a dual-action shaker. After letting the solution react for 24 h, the particles were isolated, and the supernatant was removed. The particles were then washed twice with DI water, after which DI water was added to obtain the initial concentration of 2.5 wt %. In the next step, thiourea was covalently reacted with the polyglutaraldehyde-activated particles. Specifically, 1 part 2.5 wt % particles in DI water, and 1 part 1.0 M thiourea (J. T. Baker Chemical, Phillipsburg, NJ) were mixed and placed in a water bath at 70 °C while they were shaken with a dual-action shaker. After letting the solution react for 24 h, the particles were isolated, and the supernatant was removed. The

(12) Bonen, M. R.; Garcia, A. A.; Hoffman, S. A. *J. Microbiol. Methods* **2001**, *44*, 113−120.

(13) Oleschuk, R. D.; Shultz-Lockyear, L. L.; Ning, Y.; Harrison, D. J. *Anal. Chem.* **2000**, *72*, 585−590.

(14) Kim, D.-H.; Garcia, A. A. *Biotechnol. Prog.* **1995**, *11*, 465−467.

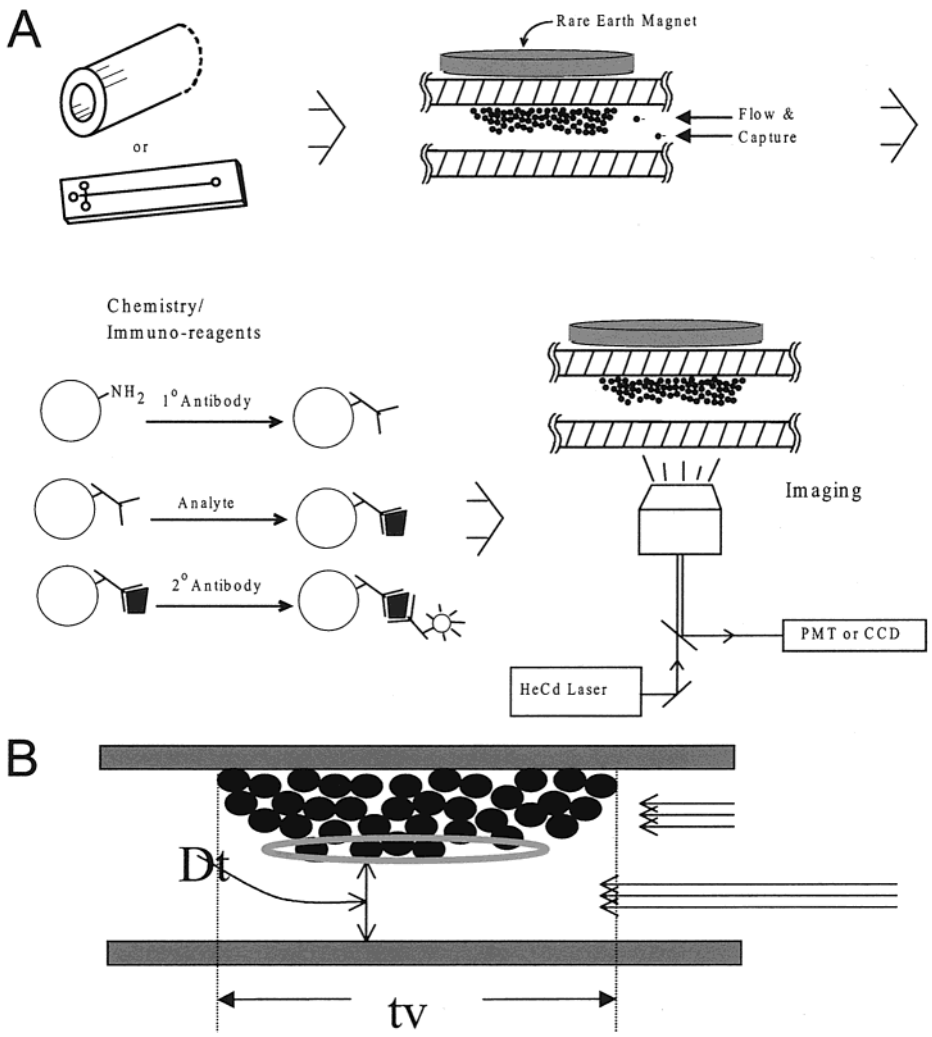

Figure 1. (A) Schematic representation of flow-based microimmunoassay system. Paramagnetic particles from a dilute solution are collected near a rare earth magnet to form a packed bed within a capillary or channel. Reagents are introduced into the packed bed to perform standard immunoassays (either heterogeneous or sandwich assay), and the bed is imaged with an epifluorescence microscope with laser-induced excitation. (B) The packed bed does not occlude the channel, and this has several ramifications for both fluid dynamics and reagent interaction and detection with the bed. Most of the volume flow passes through the open section of the channel where the reagents interact with the bed by diffusive mechanisms. As long as the bed dwell time (*tv*) is much greater than the time for radial diffusion (*Dt*) to the surface of the bed, the reagents will interact with the particle surfaces.

particles were then washed twice with DI water, after which enough DI water was once again added to yield the original 2.5 wt % particle suspension. To immobilize silver ions onto all of he available reactive thiourea groups on the paramagnetic particles and, thus, to obtain the maximum possible silver loading, a 5-fold excess was used. This excess was obtained by adding 40 μL of 1.0 M $AgNO_3$ per milliliter of a 2.5 wt % particle suspension. The resulting solution was subsequently shaken at 23 °C for 24 h using a dual-action shaker. The particles were then isolated, and the supernatant was removed. This was followed by washing the particles twice with DI water, after which enough DI water was added to obtain the initial suspension of 2.5 wt % particles. These particles were stored at 4 °C. An aliquot of 200 μL of biotinylated anti-FITC monoclonal antibodies (Sigma, Catalog No. B-0287, mouse IgG1) was added to the 2 mL of Ag-activated beads from the above steps. The suspension was mixed and shaken for 1 h at room temperature. The particles were collected with the rare earth magnet and separated from the supernatant. The particles were then resuspended in 50 mM, pH 7 phosphate buffer to make a 2-mL suspension.

Immunoassays. The paramagnetic particles were locally packed into the microchannels by the application of a strong magnetic field (2360 G) using a rare earth magnet [3/4-in.-diameter, 0.1875-in.-thick disk of NdFeB (27/30 mixed), rated at 11 lb lift (Edmund Scientific, Barrington, NJ; Catalog No. CR35-106)]. The bed was packed by placing the magnet directly over the microchannel and flowing the paramagnetic particles into the channel by applying 0.33 atm vacuum for 30 s followed by 0.10 atm vacuum for 2 min (Figure 1). A newly packed bed was used for each experiment. The particles were packed to a total bed length between 1 and 3 mm. Buffer was then flowed through the system until all components of the system were stable (packed bed, laser signal, electronic components, CCD, etc.). Fluorescence intensity was monitored at each stage of the procedure.

FITC Assay. The biotinylated anti-FITC primary antibody was immobilized by incubation with the streptavidin-labeled paramag-

netic particles for 1 h before introduction of the particles into the microchannels. A packed bed was then formed, and 20 mM NaH_2PO_4 (pH 7.0) buffer was passed through the system for 10 min to settle the particles and form a tightly packed bed. Optimal reaction time studies used 125 μM FITC exposed to a packed bed at various reaction times of 30, 60, 90, 120, 150, 180, 210, 240, 360, 480, and 600 s. Concentration studies were also performed using various concentrations (2−250 μM) of FITC. The fluorescence signal was recorded immediately before and 4 min after the FITC interaction with the bed.

PTH Assay. The labeling of the streptavidin-derivatized Dynal paramagnetic particles with biotinylated anti-PTH was performed in two ways. Initial experiments were performed by incubating the biotinylated anti-PTH (30 μL of 0.7 μg/mL) with the Dynal streptavidin-labeled paramagnetic particles (100 μL) for 1 h prior to use in which the anti-FITC antibody-to-streptavidin−biotin binding site ratio was maintained at 2:1. These labeled particles were then used to form a packed bed, as described above. Later experiments were performed by packing a bed of streptavidin-labeled paramagnetic particles and then loading primary antibody onto the packed bed by flowing biotinylated anti-PTH molecules (0.7 μg/mL) past the region for 10 min at 3 nL/s. Once a labeled, packed bed was formed by either method, diluted PBS (0.01 M, 0.04% BSA) was flushed through the packed bed region for 10 min, allowing the bed to equilibrate. PTH antigen standards (1.4 μg/L and zero standard) were then passed through the system for 10 min, followed by a 10-min buffer wash. Finally, fluorescein-labeled anti-PTH was passed through the bed for 10 min, followed by a buffer wash for an additional 10 min. Fluorescence intensity was monitored after each step, and the residual fluorescence was compared to the background levels observed prior to introduction of the labeled anti-PTH.

IL-5 Assay. Anti-IL-5 primary antibody was immobilized onto the Dynal paramagnetic beads as described in the PTH assay section. A packed bed was then formed, and blocking buffer was passed through the system for 15 min both to allow a tightly packed bed and to block any nonspecific adsorption sites using BSA. Background fluorescence was monitored until the signal stabilized, then the IL-5 sample was flowed through the bed for 30 min using a pressure difference of 0.005 atm. The bed was then washed with PBS/Tween for 5 min to remove any unbound IL-5, and images were taken to establish the background fluorescence intensity. The FITC Ab was then flowed through the bed for a period of 30 min at the same pressure driving force. The bed was then washed with PBS/Tween to remove any unbound FITC Ab, and the fluorescence intensity was quantified at 9 min from the end of the FITC Ab exposure. Concentration studies were performed using various concentrations of IL-5 standard [2 (3), 20 (1), 50 (1), and 200 (2) μg/L IL-5 (n is in parentheses)]. A signal was calculated by subtracting the background fluorescence intensity from the final fluorescence intensity, followed by subtracting the signal obtained for the corresponding control experiment in which no IL-5 was present. Control experiments that consisted of the identical protocol described above, but with no IL-5 present, were performed for each bead batch. Instead, during the sample incubation time, PBS/Tween was flowed through the bed. The fluorescence intensity was recorded as described above, and the difference was defined as the background fluorescence for this assay.

Capillaries and Channels. All experiments, except preliminary FITC-anti-FITC data sets, which were carried out in microchannels, were performed in 50-μm-diameter fused-silica capillaries (150-μm o. d.) that were purchased from Polymicro Technologies (Phoenix, AZ) and cut to 55 cm in length. The microchannels for preliminary proof of principle "on-chip" experiments were obtained from Alberta Microelectronic Corp. (recently renamed Microlyne, Edmonton, Alberta, Canada). The channels, 5.0 cm in length, were etched to 30-μm wide and 10-μm deep in Corning 0211 glass (2.5 × 7.6 cm). Similar procedures were used on both the channels and the capillaries in which the pressure/vacuum and reagents were introduced with previously described apparatus for the channels.[15] The protective polyamide coating on capillaries was removed via burning to create optical windows (~4 cm in length) for imaging. These windows were cleaned with methanol and water prior to use.

Apparatus. An Olympus IX70 inverted research microscope (Tokyo, Japan) was used for imaging. An OmNichrome model 100 HeCd laser was used as the fluorescence excitation source (442 nm, 40 mW). Image acquisition of the packed bed was performed using an RS170 CCD camera (CSI Electronics, East Hartford, CT) integrated with National Instruments LabVIEW IMAQ image acquisition software and hardware (National Instruments, Austin, TX) for which imaging programs were developed in-house. Data analysis of the images was performed by averaging a 10 × 10 pixel area (5.7 × 5.7 μm) in the middle of the microchannels followed by further data processing using MathCAD 7.0 (MathSoft, Inc., Cambridge, MA) and Excel (Microsoft Corporation, Seattle, WA) macros and subroutines that were developed in-house.

RESULTS AND DISCUSSION

The ability to reliably detect specific biomolecules quickly and sensitively at relevant concentrations in small volumes is an important goal. The combination of the high surface area and orthogonal manipulations of small-diameter paramagnetic particles, the fine fluidic controls of microdevices, and the selectivity of immunoassay complexation can result in a fast, sensitive, and reliable biochemical analysis methodology.

As a building block for the system design, paramagnetic particles are a useful tool. Particle beds can be packed, dynamically positioned, flushed, and repacked in microchannels by magnetic field manipulations.[10,16] Application of a magnetic field normal to the microchannel locally sequesters paramagnetic particles if there is a significant axial gradient.[16] This avoids some fabrication issues associated with other solid supports that do not exploit an orthogonal field.[11,13,17] In a flowing stream, a densely packed bed of small particles can be formed (Figure 2) which, in this study, was usually 1−3 mm in length. The bed region can be reproducibly formed (n > 100, multiple operators), and once formed, resists deformation throughout a particular experiment. Paramagnetic particles could be flushed out quickly once an experiment is finished by removing the magnetic field and inducing flow.

(15) Polson, N. A.; Hayes, M. A. *Anal. Chem.* **1999**, *71*, 1088−1092.
(16) Joung, J.; Shen, J.; Grodzinski, P. *IEEE Trans. Magn.* **2000**, *36*, 2012−2014.
(17) Fan, Z. H.; Mangru, S.; Granzow, R.; Heaney, P.; Ho, W.; Dong, Q.; Kumar, R. *Anal. Chem.* **1999**, *71*, 4851−4859.

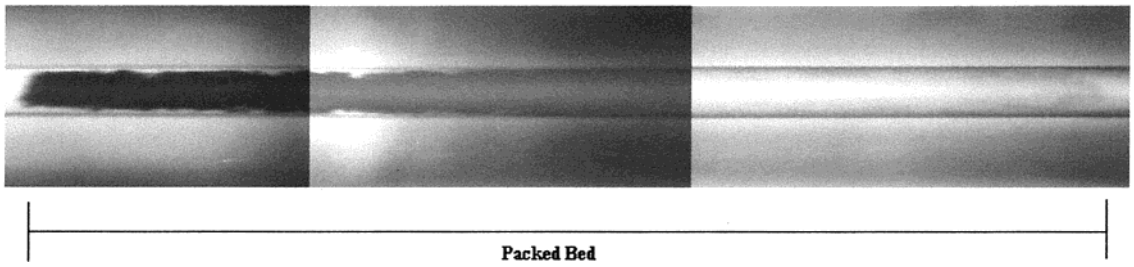

Figure 2. Composite bright field micrograph of paramagnetic particles within a 50-μm diameter capillary in the presence of a 2360 G magnetic field to form a packed bed. The packed bed is ~1.2 mm in length and ~2.4 nL in total bed volume. Packing conditions: 0.33-atm vacuum for 30 s, 0.10-atm vacuum for 120 s, 150-μm o.d./50-μm i.d. fused-silica capillary (50.8 cm length), magnet is on the far side of the outer wall of the channel.

An important feature of this particle bed/channel system is that the bed does not extend across the entire channel. This allows for high relative volume flow rates at low-pressure driving forces while still allowing sufficient time for diffusional transport to deliver antigens and antibodies to the active sites within the bed. So long as the bed dwell time, the time it takes for the fluid to traverse the particle bed at the average velocity, is much greater than the radial diffusion time, then all of the molecules in that particular volume will interact with the bed (Figure 1B). There is an additional advantage in this particular design in that most of the interaction will occur on the surface of the packed bed and can be easily detected by the inverted epifluorescence microscope system. If the bed did, in fact, extend across the entire channel, the backpressure from the packed bed of small particles would be quite large and the volume flow rates would be so diminished (~3 orders of magnitude in this design) as to add significant time to the sample exposure and washing steps. The molecules of interest would also interact to the greatest extent with the head of the packed bed and would be difficult to quantify and detect. In addition, a fully packed bed would not be stable. The forces from the backpressure would overwhelm the axial magnetic field gradients, and the bed would be flushed away.[10]

An anti-FITC−FITC complexation experiment was used to evaluate the microimmunoassay system. Although FITC is not an ideal analyte for most applications (hydrolyzes, reacts with proteins), in this instance it was sufficient to demonstrate the capabilities of the system. Control experiments to assess nonspecific binding used packed beds of paramagnetic particles without the anti-FITC antibody. After the bed was packed, a high concentration of FITC (1 mM) was flushed through the bed for 15 min, followed by a buffer rinse. Fluorescence intensity was recorded immediately after the FITC was washed from the detection zone ($t = 0$ min) and at 30 min. The fluorescence signal dropped to background levels immediately after the FITC plug exited the detection window, indicating little or no nonspecific binding took place. When anti-FITC-labeled paramagnetic particles were used, the fluorescence signal after the FITC plug exited the detection window was 15.5 ± 3.1 arbitrary (arb) units and leveled off at 8.9 ± 0.5 arb units above background levels. The high concentration caused a large photon flux that tripped the auto-iris function in the CCD, and the intensity of the signal was diminished (compared with later data); nevertheless, these control experiments indicated minimal signal for nonspecific binding of this small molecule.

Reaction time was also determined with the anti-FITC−FITC system. To investigate the reaction time for this system, an intermediate concentration of FITC (125 μM) was exposed to the bed in several separate experiments. These separate experiments indicated that interaction between anti-FITC and FITC reached 90% of the stable signal after ~180 s (Figure 3). This reaction time is much shorter than both conventional microtiter plate systems and polystyrene microchannel systems.[11,18−21] The reduced time can be understood by examining the total volume and diffusion distances within the channel/bed system. Within the bed, the particles are so closely packed that diffusion distances are trivial, whereas the entire channel volume where the bed is contained is <6 nL. The free solution diffusion constant (D) for FITC is 3.5×10^{-6} cm^2/s, so the maximum time required for a FITC molecule in solution to diffuse to the bed from anywhere in the channel radius (approximately 25 μm) is <1 s. Even though the tortuous path created by the particle bed will increase the diffusional time radially through the bed, as compared to a capillary filled with water, the particle packing density is low, offsetting the overall decrease in the effective diffusivity. More importantly, because most of the fluid flow is around the surface of the bed and because the detection system is based on the signal generated from the surface of the bed, the relevant diffusion path is actually much less than the radius of the channel. The molecules being analyzed, thus, essentially encounter free solution diffusion to reach the surface of the particle.

These diffusion factors have been recognized in immunoassay literature, and some terminology has been discussed. For example, specific interface is a term used to describe surface-area-to-volume ratio,[11] where in this system, the large surface area of the particles corresponds to a large specific interface. The specific interface approximates the number of available binding sites in the detection volume, which also has a specific term: the reaction field. In these experiments, the definition of the reaction field is somewhat complicated since the detection area and the bed volume are different. The detection volume is limited to the specific area quantitated within the fluorescence micrograph, and it is ~65 fL. The total bed volume is typically on the order of several nanoliters. The detection method is merely a sampling of the surface of the bed and not an assessment of the total binding activity in the bed volume; however, meaningful comparisons can be made to

(18) Samuel, D.; Patt, R. J.; Abeknesha, R. A. *J. Immunol. Methods* **1988**, *107*, 217−224.
(19) Rattle, S. J.; Purnell, D. R.; Williams, P. I. M.; Siddle, K.; Forrest, G. C. *Clin. Chem.* **1984**, *30*, 1457−1461.
(20) Micheel, B.; Jantscheff, P.; Bottger, V.; Scharte, G.; Kaiser, G.; Stolley, P.; Karawajew, L. *J. Immunol. Methods* **1988**, *111*, 89−94.
(21) Haaijman, J. J.; Coolen, J.; Krose, C. J. M.; Pronk, G. J.; Ming, Z. F. *Histochemistry* **1986**, *84*, 363−370.

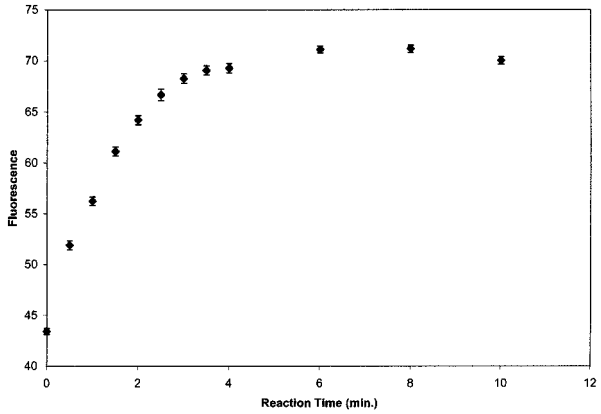

Figure 3. Reaction time for anti-FITC with FITC. A separate series of experiments was performed for each data point ($n = 5$, 2σ error bars). For each of these, data was taken 4 min after the buffer wash was initiated. Approximately 90% of the maximum signal is attained after 3 min of interaction time with the flow-based microimmunoassay.

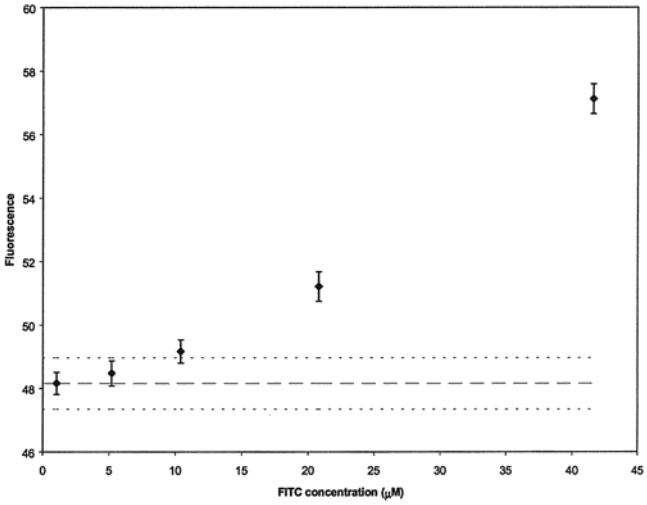

Figure 4. Calibration curve of FITC interacting with immobilized anti-FITC on paramagnetic particles ($R^2 = 0.9930$).

existing systems. Assuming a 40% void volume in the packed bed, there are ~3.4 particles in the detection area, with a representative surface area of 8.4×10^{-7} cm^2. Typical specific interface values for conventional microtiter plate immunoassays range from 1 to 13 cm^{-1}, and a recent report using polystyrene particles in microchannels reports a specific interface of 480 cm^{-1}.[18,19,21] The experimental parameters for this microimmunoassay give a specific interface of 1.3×10^4 cm^{-1}. The higher specific interface values directly relate to maximizing the speed and sensitivity of any immunoassay, including this design.

The detection limits and sensitivity of the flow-through microimmunoassay were evaluated using various concentrations of FITC, from 2 to 42 μM (Figure 4). On the basis of the background fluorescence signal, the detection limit was approximately 10 μM ($>2\sigma$). The sensitivity was ~2.5 arb units/10 μM FITC. The assay was linear at least up to 250 μM ($R^2 = 0.9930$), but it was limited at this point by the dynamic range of the optics and the CCD element employed in these preliminary experiments. Note that, as with any fluorophore that directly participates in the antibody binding, the fluorescence is quenched to some extent. On the basis of empty capillary experiments, we calculated that a little more than 50% quenching can occur as a result of the FITC/anti-FITC complexation. Binding schemes in which the optically active element of the molecule does not participate in the binding will improve quantum efficiency and, therefore, sensitivity and detection limits.

To demonstrate a heterogeneous sandwich immunoassay using real biological fluids, the parathyroid hormone (PTH) in human plasma was assayed using PTH conjugate antibodies. Biotinylated anti-PTH primary antibodies were immobilized onto the streptavidin-labeled Dynal paramagnetic particles using two different methods. The first was an off-line method in which the incubation with the antibody was performed prior to introduction of the particles into the microchannels. The second method loaded the paramagnetic particles into the microchannels to form a packed bed, and then a solution of the primary antibody was passed through to allow "on-the-fly" immobilization. Each method of primary antibody loading was assayed against a PTH standard (1.4 μg/L) and a zero standard (PTH removed from the sample by immunofiltration). Once the primary antibody was loaded onto the paramagnetic particles and a packed bed was formed, buffer and standard solutions were passed through the bed according to standard procedures.

For the off-line method, the system was tested with a 1.4 μg/L solution of PTH. After loading the PTH and washing with buffer, the absolute fluorescence signal was 30.9 ± 0.7 arb units. After the fluorescein-labeled anti-PTH* antibody was exposed to the packed bed and washed with buffer, the residual fluorescence was 39.4 ± 0.9 arb units., a 28% increase. The zero standard was then assayed, resulting in a residual fluorescence signal 5% above background levels. The on-line method resulted in a 23% rise in fluorescence for the 1.4 μg/L solution ($n = 3$) and 11% for the zero standard ($n = 2$). The on-line labeling method gave nearly the same relative rise in fluorescence, but it had an increased level of nonspecific binding for the zero standard. In terms of primary antibody consumed in each experiment, the off-line experiment required 30 μL, but the on-line experiment required only 1.8 μL, a 94% reduction of primary antibody needed for the assay. This was the only concentration tested in this preliminary work. It is on the highest end of physiologically relevant measurement, but indicates that the method is functional with real samples. The detection limit in immunoassays is typically defined by the binding thermodynamics of antibody/antigen interaction at lower concentrations. However, in this system, this binding interaction limit has not been reached, and the levels at which the normal sigmoidal roll-off is observed are buried in the background noise. So improving the sensitivity of the detection method would lower the mass and concentration detection limits. Other detection methods (chemiluminescence and enzyme approaches, for example) or improved optics and cameras should provide significantly improved detection limits to extend the range of this measurement.

This immunoassay system was further characterized with IL-5, an important cytokine associated with asthma and inflammatory responses.[22] The physiologically relevant level of IL-5 in an asthmatic mouse's lung bronchoalveolar fluid (BAL) is in the range of 2−250 μg/L. These concentrations are higher than the physiological levels of PTH and are within the range of sensitivity

(22) Lee, N. A.; Lee, J. J. *Am. J. Respir. Cell Mol. Biol.* **1997**, *16*, 497−500.

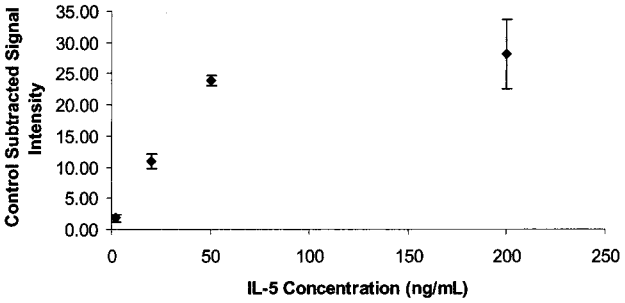

Figure 5. Calibration curve of interleukin-5 generated from a sandwich immunoassay. Biotinylated rat monoclonal anti-mouse IL-5 immobilized on paramagnetic particles, FITC conjugated rat monoclonal anti-mouse IL-5 was the detectable agent.

demonstrated in this system. Sets of standards of IL-5 ranging in concentration from 2 to 250 μg/L were tested on the flow-based microimmunoassay system (Figure 5). Although quantitative data was be collected over this range, improved detection optics and imaging are being investigated currently to allow detection of lower concentrations, with the goal of measuring the levels of IL-5 in nonasthmatic mice, which are on the order of 20 pg/mL.

The mass detection limit for this immunoassay could be extremely low for future system designs. First, the actual number of molecules detected in the present design was approximately 10 amol regardless of the total number within the bed volume. As the ability to absolutely control small amounts of paramagnetic particles in defined spaces on microfluidic devices,[16,23] very small well-defined beds will become possible. In the case shown here, the detection zone was approximately 5.7 $\times$ 5.7 μm, where the laser penetration depth is assumed to be approximately 2 μm (due to obstruction of the laser path from the particles themselves), and total bed volumes could be fabricated on this scale in the future. For the Dynal paramagnetic particles, the number of biotin binding sites (and, thus, anti-FITC binding sites) per particle is 3.9×10^6.[24] Assuming a 40% void volume, the total number of observable (line of sight) binding sites will be approximately 50% less than the total binding sites. There are $\sim 6.6 \times 10^6$ binding sites within the detection zone, some 10 attomoles.

For these assays, the amount of reagent consumed is small, some 100 to 1000 times smaller than conventional assays. This reduces the cost per assay, both in terms of reagents and waste. This format allows assays to be performed where the reagents may be available in limited quantities and is compatible with small-volume direct sampling systems. The use of paramagnetic particles also allows the independent control of the substrate beds (formed, moved, flushed) with simple magnetic field manipulations. The assay is flow-based, which allows it to be integrated into automated microfluidic devices and eliminates sample handling cost and loss of sample.

ACKNOWLEDGMENT

The authors thank James Lee, Nancy Lee, and Jeff Crosby of the Mayo Clinic, Scottsdale; and Phillip Miller, Michael Otter, and Martha Garrity of Nichols Institute Diagnostics. This work was supported in part by The Whitaker Foundation, Nichols Institute Diagnostics, The Mayo Clinic, and American Chemical Society-Petroleum Research Fund (M.A.H., no. 99-0286).

Received for review April 26, 2001. Accepted September 18, 2001.

AC0104680

(23) Hayes, M. A.; Polson, N. A.; Garcia, A. A. *Langmuir* **2001**, *17*, 2866−2871.

(24) Nichols Institute Diagnostics; Dynal: Certificate of Analysis (Product no. 351.05/06), 1998.