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Sequential Injection Immunoassay Utilizing Immunomagnetic Beads

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A novel sequential injection immunoassay (SIIA) method is described which utilizes immunomagnetic beads to investigate short-time antibody binding. The method is versatile and flexible and may therefore be adapted to many different applications. Initial results for a competitive assay are also presented. The immunomagnetic bead reactor is created within the flowing stream by retaining immunomagnetic beads with an electromagnet to form an open tube reactor. Thus, the spent beads may be discharged after each analysis. This eliminates the problems of instability of reaction surfaces and eliminates the need for additional time traditionally required for regeneration of the solid-reacting phase in order to not only save time and increase sampling frequency but also to provide each individual sampling cycle with a fresh, uniform portion of beads. The spent beads are collected off line and may be regenerated later. Short-time binding kinetic studies demonstrate linear initial binding under 1 min, which then begins to reach saturation in approximately 10 min. Competitive binding assays of monoclonal mouse IgG (MRC OX-19) to polyclonal sheep anti-mouse IgG immobilized to the immunomagnetic beads show reproducible linear displacement in 30-120-s reactions. Fluorescence detection is utilized with a detection limit of 155 ng/mL, and since the reaction time is typically 2 min or shorter, the sampling frequency is 30 samples/h.

INTRODUCTION

Flow injection analysis (FIA) and immunology have recently been combined to create flow injection immunoassays (FIIA).[1-11] The small volumes required, reduced sampling handling, and precise reproducibility of FIA have been shown to be useful for improving cumbersome, time-consuming, labor intensive, and semiquantitative traditional immunoassays. FIIA systems often utilize an immobilized reactor that requires regeneration after each analysis to renew the reaction surface. This often slows the analysis and reduces precision due to changes brought on the reaction surface by repeated regeneration.

In the present investigation, we have applied sequential injection to immunoassays (SIIA) to develop a novel system (Figure 1A), which allows subminute kinetic studies of immunochemical systems utilizing a renewable reaction surface formed with immunomagnetic beads. Immunomagnetic beads have been used widely in previous batch clinical applications. (Dynal lists over 300 publications.) A typical SIIA sequence, illustrated in Figure 1B, is as follows: (1) immunomagnetic beads which are coated with antibodies against the protein are aspirated into the reaction coil (RC), which is located within an electromagnetic field. Turning on the electromagnetic traps the immunomagnetic beads within the coil to form an immobilized reaction surface. This surface replaces the immobilized antibody columns more commonly utilized with a renewable reactor. Next, the immobilized bead bed is washed to assure no untrapped beads remain in the reaction coil. (2) The protein which has been labeled with a fluorescent tag is then aspirated into the reaction coil, and the flow is stopped for a specified contact time. This contact time can vary to cover a dynamic range from initial binding to equilibrium. The precise computer timing of SI allows control of this timing to 0.1 s to investigate the kinetics of binding. (3) Following the stopped flow, a flow reversal discharges the unbound portion of the sample mixture to the detector to determine the amount of unbound labeled reagent present. This yields a signal at the detector which can be related to the protein concentration. (4) Finally, the electromagnetic field is turned off and the reactor contents are flushed to waste. The system is then ready for another assay. Obviously this technique is quite flexible and should be adaptable to most of the detection schemes which have been utilized for clinical immunoassays.

The use of magnetic particles as a surface for immunomagnetic binding has been previously investigated in a continuous flow model.[12] A radioimmunoassay technique developed by Forrest utilized a magnetic cellulose solid phase for binding. AutoAnalyzer modules made up the system which allowed 10-min incubation times for reaction. However, while AutoAnalyzer system offered a fully automated immunoassay, the SIIA system described in this paper remains unique since it allows one to exploit fast reaction kinetics, in the range of 5 s up to 2 min. In our opinion, such a short reaction time will lead to higher selectivity by avoiding the nonselective binding which occurs at longer contact times. A discussion of the basic differences between continuous flow analyzers and FIA-based techniques can be found in ref 13. The SIIA system described utilizes commercially available magnetic beads, which may be coated with a variety of antibodies, to study the short time kinetics of binding. Initial results of a competitive immunoassay are also given. However,

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(11) De Alwis, W. U.; Wilson, G. S. *Anal. Chem.* **1985**, *57*, 2754-2756.
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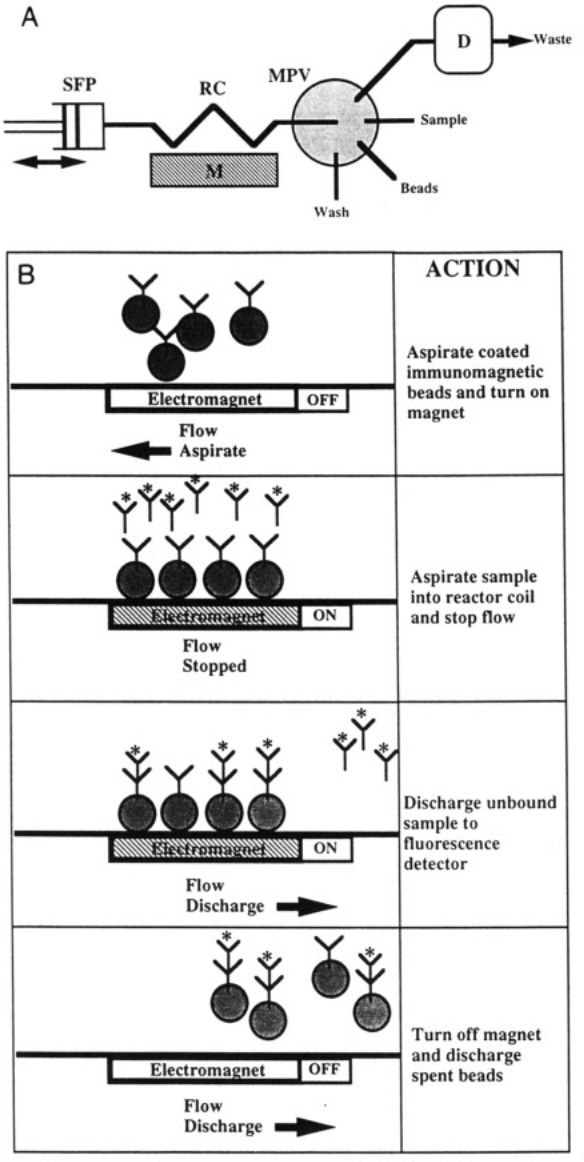


Figure 1. (A) SI system which consists of a sinusoidal flow pump (SFP), electromagnet (M), 8-port multiposition valve (MPV), and fluorescence detector (D). The reactor channel contains a reaction coil (RC) which is located within the magnetic field. (B) Illustration of the steps in SIIA: (1) aspirate the coated immunomagnetic beads and turn on the magnetic field to immobilize the beads, (2) aspirate the labeled sample into the reactor coil and stop the flow to allow for sufficient contact time, (3) inject the unbound fluorescing portion into the detector, and (4) release and discharge the spent beads to be regenerated off-line.

in its current state, there is no advantage in sensitivity to the proposed method. Instead we wish to focus this discussion on the usefulness of sequential injection and a renewable reaction surface to carry out fast binding and study initial binding characteristics. For assays in which added sensitivity is required, one may wish to modify the assay to utilize other detection schemes such as chemiluminescence or liposome-based immunoassays which increase sensitivity by several orders of magnitude. Studies of noncompetitive assay techniques utilizing a renewable reaction surface formed within the view of the detector are currently under investigation in our laboratories. We believe that optimization of this technique for sensitivity will provide an assay well suited to clinical applications.

EXPERIMENTAL SECTION

Apparatus. The system consists of an Alitea S2 dual piston cam-driven sinusoidal flow pump (Alitea USA, P.O. Box 26, Medina, WA 98093-0026), and the maximum flow rate was set at 0.50 mL/min unless otherwise indicated. A Valco 8-position multiposition valve (Valco Instruments, P.O. Box 55603, Houston, TX 77255) was used for selection of the solution to be aspirated (see Figure 1A), and all lines consisted of 0.5-mm-i.d. Microline tubing, except the aspiration line between the valve and the electromagnet, which was 1.0-mm-i.d. Microline tubing. The detector was a Hewlett-Packard Model 1046A programmable fluorescence detector (Hewlett-Packard, P.O. Box 3919, Los Angeles, CA 91604) with dual monochromators for excitation (488 nm) and emission (520 nm) settings. The source is a xenon arc lamp operated at 55 Hz. The gain of the detector was set at 16 for most kinetic work, and an internal boxcar filter was set at 125 ms averaging of the signal before sending it to the analog output. The flow-through detector, designed by the manufacturer for HPLC work, was replaced by the flow cell shown in Figure 2. The modified flow cell avoids high back pressure by using Microline tubing of 0.5-mm i.d. connected directly to the quartz tubing via ferrills, which serve to form the seals for the cell. Note that the flow path is thus essentially straight with flow from the bottom up. Plugs were also inserted over the tubing to tightly fit into the cell to reduce the dead volume, these Tygon plugs were placed just beyond the range of the light path, and the cell volume is approximately 30 μL. The detector light path was not modified, yet it was observed that some sensitivity was lost because of the cell geometry. However, it remains suitably high, and the flow arrangement is more desirable for the present application.

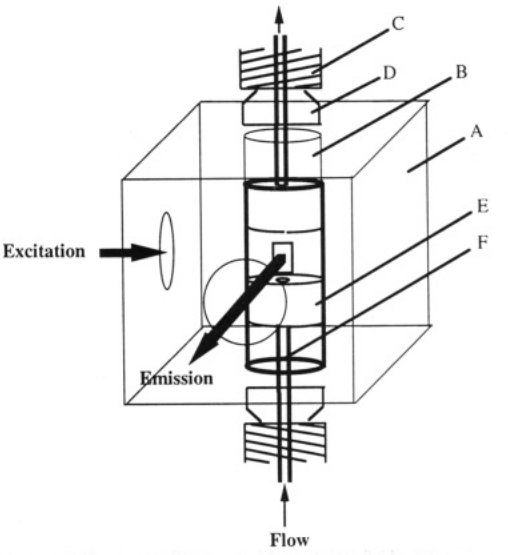


Figure 2. Modified flow cell for Hewlett-Packard fluorescence detector. The cell utilizes all the mounting features originally present to correctly position the modified cell. The cell consists of a block of delrin (A), which has a vertical hole through it for placement of the quartz flow cell (B). Excitation and emission paths are identical to those of the original cell and fitted with the same optics. The flow cell is sealed and held in place using Upchurch fittings (C), and ferrills (D) which serve to apply the pressure to form the seal. Tygon tubing plugs (E) are utilized to minimize cell volume, with the Microline tubing (F) inserted into the opening.

Two types of electromagnet designs illustrated in Figure 3 were investigated in this study. The first electromagnet (A) was made by modifying a transformer to create the field. This was accomplished by cutting a 3-mm channel in one of the arms of the transformer and supplying it with a constant dc voltage of 1–7 V. Above this range, the transformer began to noticeably heat. The dimensions of the face were 10 × 40 mm. A flow cell with a meandered flow path was placed in this channel and had a path length of 16 cm or a volume within the magnetic field of 96 μL. The flow cell consisted of 1.0-mm-i.d. Microline tubing, which was held in position with a block of delrin plastic. The second electromagnet (B) was made by wrapping a 10 × 100 × 40 mm block of soft steel with fine magnetic wire. This arrangement has the advantage of allowing one to view the behavior of the beads within the field. The magnet had a resistance of 113 Ω and was supplied with 30 dc V, which did not

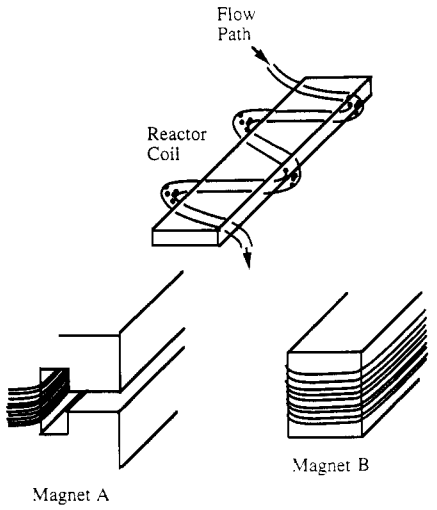


Figure 3. Magnetic coil designs of renewable reactors based on periodic retaining of immunomagnetic beads. The flow path for magnet A is placed between two 10 × 40-mm faces 3-mm apart. Magnet B allows viewing of the "column" by placing the flow path on top of the 10 × 100-mm face of the magnet.

result in noticeable heating. Power was supplied to the magnets via a B&K Precision Model 1610 dc power supply (Division of Maxtec International, 6460 W. Cortland, Chicago, IL 60635). Other magnet configurations are currently being investigated.

System Control and Data Handling. The timing and control of the SIIA system was carried out with an IBM PC through an RTD interface board. Software for operating the pump, valve, and magnet were written in house in the MATLAB environment. Data were acquired at 10 Hz, and the signal was filtered using a Butterworth low-pass filter.

Immunomagnetic Beads and Antibodies. Antibody-coated beads were obtained from Dynal (Dynal Inc., 475 Northern Blvd., Great Neck, NY 11021) as sheep anti-mouse IgG coated Dynabeads M-450. These beads are monosized, superparamagnetic, polystyrene beads with affinity-purified sheep anti-mouse IgG covalently bound to the surface. M-450 beads are 4.5 μm in diameter and are supplied as a suspension of 4×10^8 beads/mL (30 mg/mL) in phosphate-buffered saline (PBS), pH 7.4, with 1% bovine serum albumin (BSA) and 0.02% NaN_3. It is recommended by the manufacturer that 0.2–2.0 μg/mL of the primary antibody of interest be mixed with 1 mg/mL of the M-450 beads. The 4.5-μm beads are recommended for cell binding; however, this size was chosen over the 2.8-μm beads for the higher magnetic susceptability of the larger size, which allows for better control in the flow stream. The beads were diluted 50× with PBS, and 25-μL injections were used for the kinetic study assay. The beads were maintained as a suspension for the assays. The competitive immunoassay utilized a PBS/BSA buffer with NaN_3 and Tween 20. Uncoated M-450 beads were also obtained from Dynal and were used for blanks and bead retention studies. For the latter work, the fluorescence detector was set at zero-order excitation and 400-nm emission to detect light scattering caused by the beads.

Mouse monoclonal IgG, OX-19, against an antigen expressed on rat thymocytes and T lymphocytes was obtained either as a purified IgG or as fluorescein–isothiocyanate- (FITC-) conjugated IgG from Serotec (Kidlington, Oxford, England) for use as models to study direct binding and competitive binding to Dynabeads using the proposed system. The FITC-OX-19 was supplied in PBS, pH 7.2, at a concentration of 1.0 mg/mL. For most assays, a 200× dilution with PBS was used to give 5 μg/mL of the labeled antibody. Unlabeled OX-19 was supplied as ascites fluid at a concentration of total protein of 38.6 mg/mL as determined by the biuret method with mouse IgG1 at a concentration of 6.8 mg/mL as determined by radial immunodiffusion and was used at concentrations ranging from 100 ng/mL to 10 μg/mL total protein. The sample injection volume was 25 μL.

The PBS buffer was made and stored as a 10× stock solution of 80 g of NaCl, 2 g of KCl, 21.6 g of $Na_2HPO_4 \cdot 7H_2O$, and 2 g of KH_2PO_4 made up to 1 L and containing 1% NaN_3. Working solutions were adjusted to pH 7.4 with HCl. PBS/BSA/Tween 20 solutions contained 0.1% BSA and 0.25% Tween 20. All reagents were stored at 4 °C when not in use.

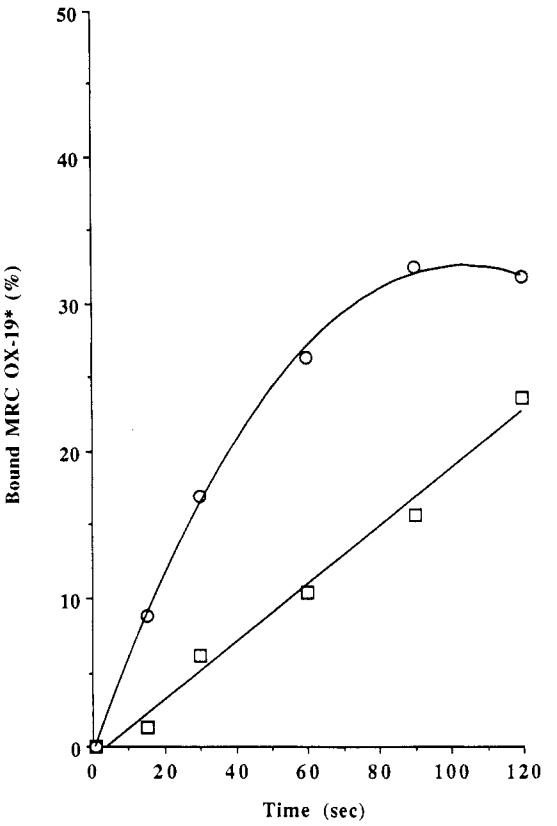


Figure 4. Plot of the percent of FITC-labeled OX-19 directly bound to the immunomagnetic beads versus contact time in seconds for cell A. (box) 10 μg/mL OX-19; (O) 1 μg/mL OX-19.

RESULTS AND DISCUSSION

Binding Kinetics. The SIIA magnetic system shown in Figure 1A was used in all experiments. The reaction coil, situated in the magnetic field, is placed between the selector valve and the pump line and is positioned as closely to the valve as possible to prevent dispersion and spreading of the sample zone beyond the boundaries of the cell. The Alitea double-piston sinusoidal flow pump was equipped with a 1-mL syringe (instead of the standard 5 mL). This allows for more precise control of the volumes of reagents, which can be as small as 1–2 μL.

The initial binding of OX-19 at 10 and 1 μg/mL, respectively, is shown in Figure 4 for magnetic coil A. The short-time binding kinetics in a range of 1–120 s demonstrate that the lower concentration of antibody appears to bind more rapidly, to reach 33% bound in 90 s. The higher concentration exhibits a linear increase in percent bound within this 120-s window and was found to reach the same 33% level in 240 s, with a gradual increase in binding to 48% bound after 480 s of contact time. Control experiments performed in the absence of beads within this 2-min range showed no appreciable loss of signal. This control was carried out to assure no axial dispersion of adsorption of the protein to tubing walls occurred during the stop time. As a blank, the uncoated beads were substituted for the sheep anti-mouse IgG-coated beads. These beads contain a much higher surface area of polystyrene for potential nonspecific adsorption and, thus, should be considered as an upper limit for nonspecific binding. Given 90 s of contact, there was a 5.47 ± 1.15% loss of signal due to nonspecific binding to the bead surface.

The relative standard deviation for measurements with the coated beads for a given contact time was 3.8% (n = 5),

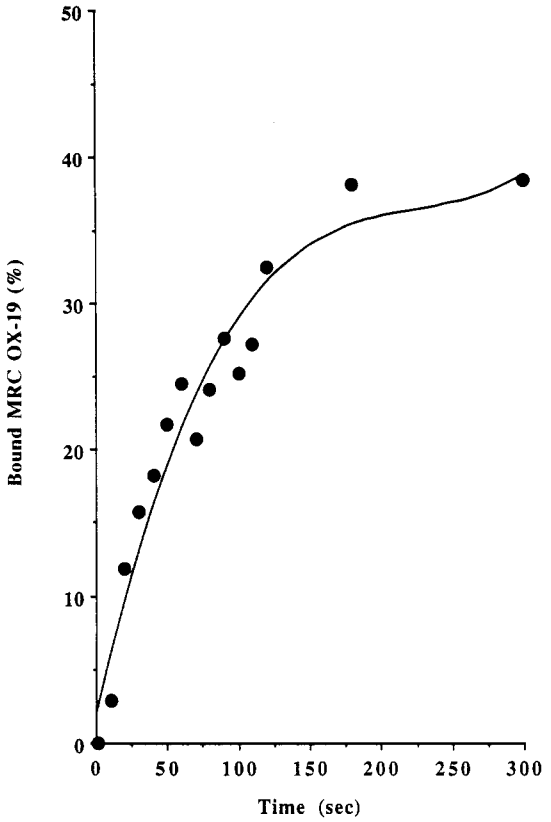


Figure 5. Plot of the percent of FITC-labeled OX-19 directly bound to the immunomagnetic beads versus contact time in seconds for cell B. The OX-19 concentration was 5 μg/mL.

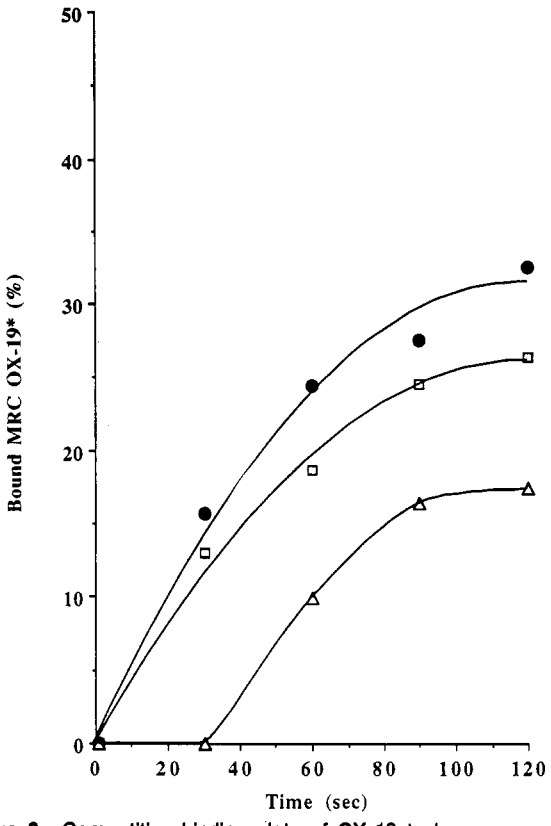


Figure 6. Competitive binding plots of OX-19 to immunomagnetic beads. Samples of OX-19 at concentrations of (●) no competing OX-19, (□) 17.5 ng/mL (100 ng/mL total protein), and (△) 1.75 μg/mL (10 μg/mL total protein) competing OX-19. All samples contain 5 μg/mL FITC labeled OX-19.

based upon a 5-μg/mL sample of OX-19 for a contact time of 90 s. The limit of detection of 155 ng/mL is based upon twice the standard deviation of a blank PBS solution injected into the system with a contact time of 1 s (n = 10). The interassay coefficient of variation[14] was 7% based upon identical assays carried out 5 days apart. The intraassay CV was 3%. Both calculations are based on assays carried out using 5 μg/mL OX-19 with contact times varying from 1 to 120 s.

Magnetic coil B was also used to study binding kinetics (Figure 5). The detailed plot of the kinetics of 5 μg/mL OX-19 FITC-labeled confirms the binding rates obtained with magnet A. Magnet B allows viewing of the beads, to verify the correct positioning of the beads during contact time. The short-time kinetic data, which can be obtained with SIIA, are clearly evident.

This data reflects the binding rate of a mouse protein to the immobilized sheep anti-mouse antibodies of the beads. It allows a good indication of the activity of the antibodies following covalent binding to the surface of the beads, thus offering a way to determine the behavior of the active surface prior to applying it to a separation or immunoassay technique. Since Dynabeads can be purchased as uncoated or tosyl-activated beads, they are quite useful for attaching a wide variety of antibodies to the surface. This SIIA system can then be utilized to investigate the characteristics of the antibody once it has been bound to the beads. The activity and binding kinetics can quickly be determined for a wide variety of antibodies. Also, since many cell separation techniques apply a primary antibody to the antibody coated on the bead surface, this antibody complex may be analyzed by the same method.

(14) Lark, P. D.; Craven, B. R.; Bosworth, R. G. L. *The Handling of Chemical Data*; Pergamon Press: Oxford, 1968.

Competitive Binding. In order to carry out an immunoassay of a serum sample, a detectable change in signal intensity needs to be correlated with the presence of the antibody or antigen in the sample. The SIIA system described for carrying out kinetic studies was applied to this by "spiking" an unlabeled antibody with a known quantity of an identical FITC-labeled antibody. First, the beads are placed into the magnetic field and held, and next the spiked sample is introduced onto the beads. If the sample contains little antibody, then the spiked labeled antibody will undergo maximum binding and a small resultant signal will occur at the detector. On the other hand, if a large amount of antibody exists in the sample, then many of the active sites which would normally bind the labeled antibody will be occupied, and a large signal will be detected. This competitive assay is optimized if there is only a slight excess of labeled antibodies for the sites available. However, it is inherently not as sensitive as the popular sandwich techniques, which use an excess of both labeled and unlabeled antibodies to drive the reaction to the maximum bound state. Figure 6 shows the results for two different concentrations of competing antibody spiked with 5 μg/mL FITC-labeled antibody. The upper curve is for only labeled antibody and displays the expected binding behavior. The other two curves have additional unlabeled OX-19 present at concentraions of 17.5 ng/mL and 1.75 μg/mL, which results in a decrease in the binding of the labeled OX-19. Concentrations of 1.75 μg/mL result in a 16% change in signal within the first 30 s of competitive binding; however, at this time, there is only a 2.8% difference for the 17.5 ng/mL OX-19 sample. The lower concentration can be distinguished from the sample containing no unlabeled OX-19; however, it lies slightly below the calculated detection limit of 155 ng/mL.

Another competitive immunoassay scheme was also investigated in which the beads and sample were sequentially

Table I[a]

no.	mean[b]	time[c]	OX-19[d]	magnet[e]	% bound
1	+	−	−	−	10.3
2	+	+	−	−	14.8
3	+	−	+	−	11.4
4	+	+	+	−	12.5
5	+	−	−	+	9.4
6	+	+	−	+	14.1
7	+	−	+	+	11.6
8	+	+	+	+	15.7

[a] Time, +60, −120 s. OX-19, +0.88, −1.75 μg/mL. Magnet, +off, −on. [b] Effect, 12.48 ± 0.84. [c] Effect, 3.57 ± 1.67. [d] Effect, 8.05 ± 1.67. [e] Effect, 0.45 ± 1.67.

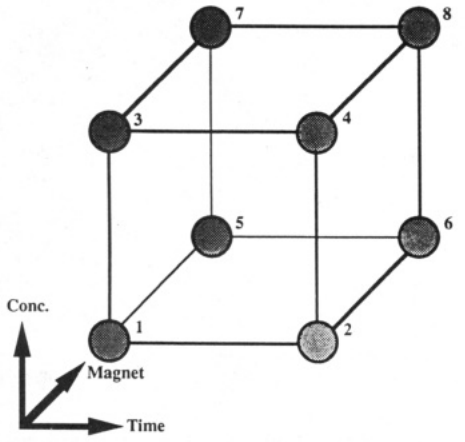
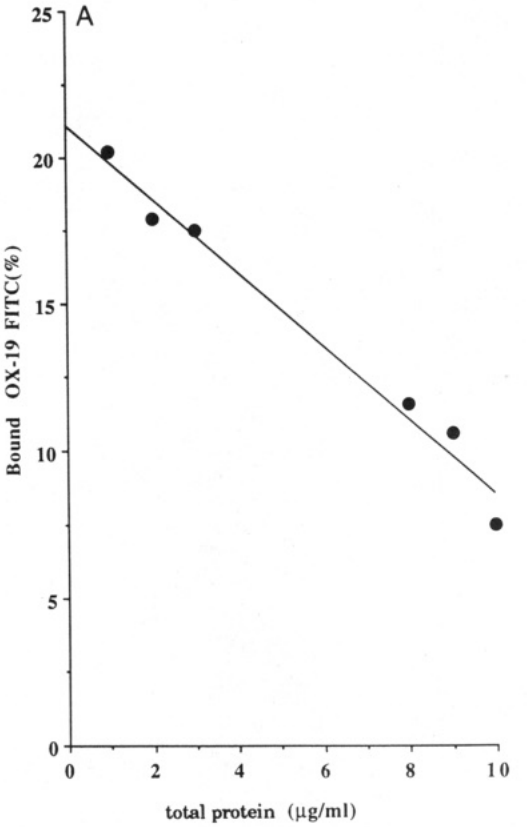


Figure 7. Two-level factorial design illustrating the format for data in Table I.

aspirated into the reaction coil and merged in the flowing stream. The beads were washed prior to the assay in the PBS/BSA solution described in the Experimental Section to help assure no unbound sheep anti-mouse IgG was present and then were diluted to a final concentration of 1 mg/mL. PBS/BSA was also used as the carrier, and Tween 20 was added to help the beads move in solution. Following aspiration, the flow was again stopped to allow the specified contact time. Next, the magnetic field was turned on and the unbound portion injected through the detector. Once the unbound portion had passed, the field was turned off and the beads containing the bound portion of spiked sample were released. This method increased the speed of the assay and allowed the bound portion to be detected. Experiments carried out with 1.75 μg/mL OX-19, FITC-labeled, over a 120-s time range showed the expected decrease in the unbound portion. However, no signal was obtained from the beads as they passed through the detector, although calculated amounts of labeled antibody at 10× the detection limit were present. One possible explanation for this is the quenching of the fluorescence as the labeled protein is bound. However, as recently discussed,[15] there is a lack of sensitivity for 90° fluorescence measurements on the surface of particles. Therefore, detection of the bound portion was not possible for the current arrangement.

This behavior was exploited by investigating if detection of the unbound portion would still be possible in the presence of the beads. Since the method looks at a competitive 1:1 ratio, an X versus $(1 - X)$ relationship exists between the bound and unbound portions, and with proper experimental design, good sensitivity can be achieved with the indirect method. The high precision of the flow injection technique is beneficial for such measurements. Table I shows the results

(15) Alvanger, T.; Balcavage, W. X.; Ghosh, S.; Geib, R. W.; Goff, C.; Laherty, R. F.; King, M.; Personett, D. *Am. Lab.* **1991**, *11*, 21–27.



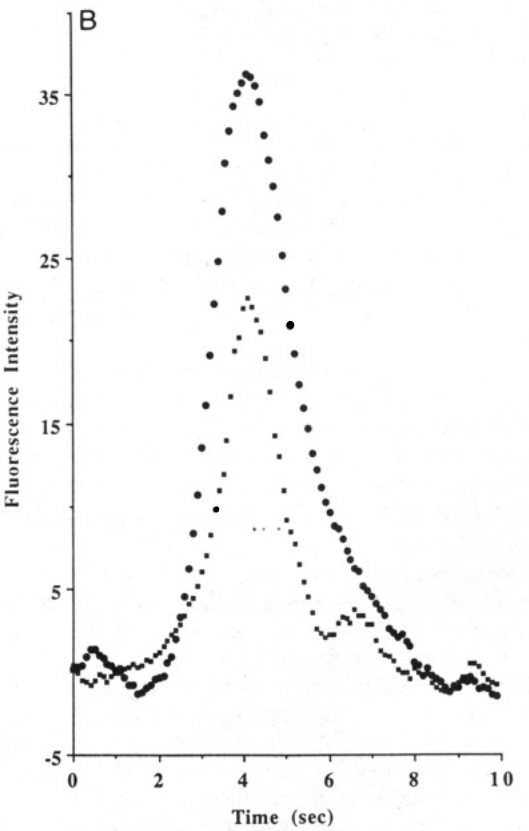


Figure 8. (A) Calibration line for the competitive immunoassay for total protein ranging from 1 to 10 μg/mL with 5 μg/mL FITC-labeled OX-19 and a contact time of 90 s; the measurements were achieved in the presence of released beads. (B) Illustration of the sample (●) and bead (□) peak position for the competitive assay. The peak for the beads was obtained by a light-scatter measurement.

of a two-level factorial design carried out which investigated if the retention of the beads was necessary. The factorial design is illustrated in Figure 7. The main effects of reaction

time, secondary antibody concentration, and retention of the beads were calculated via the Yates Algorithm[16] and show a low value for the effect of retaining or releasing the beads. The error term was calculated based on the time, concentration, beads, correlation effect, and value. Based on this low value, a series of competitive assays was carried out not retaining the beads. The results of varying the total protein over a range from 1 to 10 μg/mL (OX-19, 17.5 ng/mL to 1.75 μg/mL) with 2 mg/mL sheep anti-mouse IgG-coated beads are shown in Figure 8A. The percent bound ranges from 7.5 to 20.2% for 90 s of contact time. The correlation coefficient (r^2) is 0.98 with a relative standard deviation (RSD) for the assay of 2.1%. Figure 8B illustrates the peaks for both the sample and the beads. So while the two remain overlapped, detection is possible with less than 60 s of liquid manipulation required for this competitive assay.

CONCLUSION

Sequential injection offers several advantages for immunoassays. The highly reproducible timing obtained with sequential injection allows for accurate analysis that can extend into nonequilibrium measurements in a very short time frame not generally considered or achieved by a batch (well) technique. Sequential injection accelerates sample handling, which in batch methods is too slow thus preventing the utilization of short-time kinetics. Stop-flow techniques enhance the usefulness of SIA by allowing well-controlled contact times between antibody and antigen, which can range from only a fraction of a second into the traditional equilibrium time frame. The use of the syringe pump in the SIIA system adds an element of ruggedness over usual FIA in that a syringe replaces the peristaltic pump, in which the tubes stretch and wear over time, giving changing results. Sample volumes injected are very small, and there is also a very low reagent consumption. This becomes an important consideration when using purified antibodies.

Flow injection has been applied to many immunoassays in the past and has proven to yield reproducible results. Many of these techniques rely on a microreactor that contains an immobilized antibody. Detection relies on the sample analyte of interest binding to the immobilized antibody, which allows for detection by several different techniques. Then following the detection, conditions within the column need to be modified to break the bond formed to remove all bound material from the microreactor. At best, this process is time-consuming and slows the sampling frequency. In addition, it may be difficult to break the antibody bond without affecting the antibody itself or the bond immobilizing it. The conditions required to regenerate the column as quickly as possible are often sufficiently harsh that the activity of the reaction surface is affected. Thus, it is difficult to obtain both reproducibility and the high sampling frequency desired for most assays. The advantages of renewable reaction surface for each assay are clear. New beads are injected for each assay, and the sampling frequency is not decreased as it is when regeneration is a part of each measuring cycle. In addition, the technique is sufficiently flexible that most of the detection schemes can be applied.

The SIIA system can be utilized to characterize antibodies which have been bound to the surface of the magnetic beads for use in immunoassays. Information concerning the activity and kinetics of binding is quickly obtainable to help judge the behavior of an immobilized antibody. In addition, the same system without modification may be used to carry out a competitive assay. Current investigations under way in our laboratories involve the expansion of the use of magnetic beads with flow injection to develop systems capable of sandwich-type immunoassays which will increase the sensitivity of the assay.

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