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Use of Excess Solid-Phase Capacity in Immunoassays: Advantages for Semicontinuous, Near-Real-Time Measurements and for Analysis of Matrix Effects

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A flow-based immunoassay system using solid-phase particles with high binding capacity was used for semicontinuous, near-real-time, measurement of 17β-estradiol (E2). The high binding capacity of the solid phase was exploited to enable (i) a quantitative determination of E2 concentration, based on rate of accumulation of fluorescently labeled anti-E2 antibody on the solid phase, and (ii) the use of a single solid phase for more than a dozen competitive binding measurements. The high binding capacity of the solid phase also permitted the immobilization of a second capture antigen. Biotin was immobilized as a second antigen and used to evaluate a biotin anti-biotin system as a control for matrix effects in the E2 immunoassay. In phosphate-buffered saline, E2 could be quantified (in the range of 10−1000 pM) by using either the summation or ratio of the signals from the labeled anti-E2 and anti-biotin antibody in the presence of biotin at a constant concentration. The same referencing system was applied to estimate the matrix effects in selected environmental samples. Matrix effects that inhibited the binding of the anti-E2 antibody to the solid phase led to false positive responses, but these matrix effects could be identified and partially corrected using the response from the anti-biotin antibody.

Immunoassays have been widely applied in clinical diagnostics for many years.[1] More recently, immunoassays for environmental analytes have also become commercially available; however, some of the problems associated with environmental immunoassays have only been partially solved. In particular, the exact composition of the matrix of environmental samples is usually unknown and can vary widely from sample to sample, even from the same source. In many cases, the sample matrix itself can affect the binding of the antibody, either to the antigen in the sample or to the solid-phase support, or both, leading to errors in determination.[2,3] In addition, in applications such as monitoring of wastewater at treatment plants it may be desirable to have a system both rugged enough for the operating environment and capable of continuous automatic operation.

Endocrine disrupting chemicals, in particular those that mimic estrogen or interfere with estrogen's normal expression and effects, continue to receive a great deal of attention as environmental pollutants.[4−6] The evidence that xenobiotic (nonbiological) endocrine disruptors present in the environment can influence the sex distribution and reproductive success of various types of marine and terrestrial animals is well established.[7−9] While the occurrence of deleterious effects from environmentally occurring endogenous estrogens is still under active investigation, it has been reported that as much as 88−99% of the estrogenic activity of treated wastewater is due to the combination of estradiol and the synthetic estrogen ethynylestradiol.[4] To more fully monitor the occurrence of endocrine disrupting chemicals in waste and surface water, rapid, simple, and sensitive detection methods for the various members of this class of substances are required.

Recently, this laboratory has shown that theoretical limits of detection for estradiol and estriol can be readily achieved in a flow-based immunoassay system employing plastic particles as the solid phase (the KinExA system).[10] Another recent report describes combining several anti-estradiol or anti-estriol antibodies in a single assay to extend the dynamic range or allow the

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detection of multiple analytes in a single assay.[11] In this report, we describe the use of the excess solid-phase capacity of the KinExA system to make multiple sequential measurements on a single solid phase. In conjunction with a modification of KinExA to use binding slopes, this enables semicontinuous real-time measurements. In addition, we report on the inclusion of a second antibody as a reference and our attempt to use this reference to correct for some of the matrix effects present in real environmental samples.

MATERIALS AND METHODS

Antibodies and Chemicals. Monoclonal anti-estradiol (anti-E2, Catalog No. A54060069P) was purchased from BiosPacific Inc. (Emeryville, CA). Cy5 conjugated monoclonal anti-biotin antibody (Catalog No. 200-172-096) came from Jackson ImmunoResearch Laboratories (West Grove, PA.). β-Estradiol 6 (carboxymethyl)-oxime BSA (E2-BSA, Catalog No. E5630) and biotin-BSA (Catalog No. A8549) both came from Sigma Chemical (St. Louis, MO). Poly(methyl methacrylate) (PMMA) particles (100-μm diameter, Catalog No. 440107) were purchased from Sapidyne Instruments Inc. (Boise, ID). 17β-Estradiol (Catalog No. 052-04041) and biotin (Catalog No. 023-08711) came from Wako Pure Chemical Industries (Osaka, Japan). Cy5 antibody labeling kit (Catalog No. PA35000) came from Amersham Biosciences (Little Chalfont Buckinghamshire, U.K.). The anti-estradiol (anti-E2) antibody was labeled with Cy5 using the Amersham labeling kit following the recommended procedures included with the kit.

Antigen Immobilization. PMMA particles were adsorption coated with protein conjugate (E2-BSA or biotin-BSA) by suspending 200 mg of dry weight of particles in 1 mL of phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, with 1.5 mM NaN_3 as a preservative) containing 100 μg of conjugate. This mixture was rotated at 37 °C for 2 h. At the end of this time, 10 mg of BSA was added (to block nonspecific binding) and the particles were rotated at 37 °C for another hour. Coated particles were stored in the refrigerator and diluted to 30 mL with PBS prior to use.

Environmental Samples. The surface waters used were sampled from Tega Lake and Tone River, communal water sources in the Chiba prefecture of Japan. Environmental samples were filtered through 0.2-μm filters and frozen at −20 °C until just before use. When environmental samples were spiked, antibodies and antigens were prepared 4 times more concentrated than the desired final concentration in PBS, which was prepared 2 times more concentrated than usual. Spiking was accomplished by adding one volume of antibody and one volume of antigen to two volumes of sample. This procedure yielded an environmental sample buffered with PBS and containing the desired final concentrations of antibody and antigen. When signals from both PBS and the environmental sample were compared on the same solid phase, PBS samples were prepared in the same way (substituting deionized water for environmental sample) to ensure the same concentrations of antibody, antigen, and buffer components.

Assay. The KinExA 3000 instrument (Sapidyne Instruments Inc. Boise, ID) was used as the immunoassay platform. Briefly, this consists of a capillary flow cell in a filter fluorometer with the means to automatically replace immunoactive solid-phase particles and flow samples. In use, a batch of particles was coated with antigen conjugate (described above) and packed into the capillary flow cell. A reaction mixture containing Cy5-labeled antibody(s) and a specified concentration of antigen(s) (see Discussion) was rapidly passed through the flow cell containing the solid phase. As established previously,[10,12] binding to the solid-phase antigen conjugate is proportional to the concentration of free (i.e., not complexed with solution-phase antigen) antibody in the reaction mixture. Antigen present in the reaction mixture (if any) reduces the concentration of free antibody in accordance with the laws of mass action, allowing quantitative determination of the antigen concentration. Fluorescent signals observed during the flow of the reaction mixture through the flow cell contain a component due to fluorescent antibody bound to the solid phase, as well as a component due to fluorescent antibody in solution in the interstitial volume between the particles. Fluorescent antibody accumulates on the solid phase while the constant flow continuously replenishes the interstitial solution, thus keeping its concentration constant. The fluorescent signal (recorded as volts of response of the fluorescent detector) was recorded once per second and slopes (reflecting accumulation of fluorescently labeled antibody on the antigen-coated beads) were calculated from best-fit straight lines to a plot of fluorescent signal versus time during the flow of labeled antibody through the flow cell. More detailed descriptions of the KinExA instrument can be found elsewhere.[10−12]

RESULTS AND DISCUSSION

The KinExA instrument is composed of an arrangement of tubing, connectors, valves, syringes, and pumps; the purpose of these is to deliver accurate quantities of soluble or suspended reagents to the observation cell of the fluorometer. This observation cell is fitted with a microporous screen, and uniform particles larger than the pore size of the screen are deposited above the screen into a packed bed. A small volume of the analyte/fluorescently labeled antibody mixture (typically 0.5−5 mL) is then percolated through the packed bed of antigen-coated microbeads under negative pressure. Labeled antibodies with unoccupied antigen-binding sites are available to bind to the immobilized antigen coated on the surface of the beads; antibodies with both binding sites occupied with soluble ligand are not. Exposure of the soluble binding mixture to the immobilized antigen is sufficiently brief (~480 ms for a flow rate of 0.5 mL/min) to ensure that negligible dissociation of the soluble antigen−antibody complex occurs during the time of exposure to the beads. Those antibodies with occupied binding sites are thus kinetically excluded from interactions with the immobilized antigen. A previous report[10] demonstrated that, when a reaction mixture of antibody and antigen was drawn past the packed beads in the KinExA flow cell, only a small constant fraction (1.4%) of the free (not bound to solution antigen) antibody in a mixture could be captured on antigen-coated beads. The referenced study established that this was true over a wide range of antibody concentrations and despite the fact that the solid-phase capacity was in significant excess. These three facts, that only free antibody was captured, that the capture fraction was small and constant, and that there was a substantial excess solid-phase capacity, suggested that the KinExA assay format may be useful as a semicontinuous

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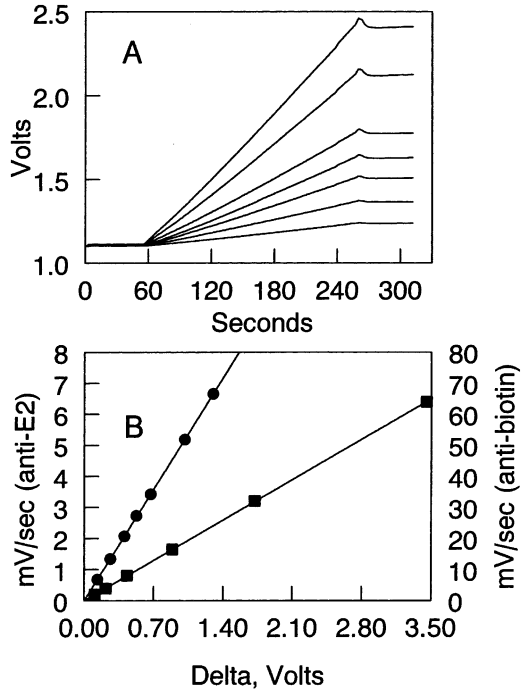

Figure 1. Volts versus free antibody concentration. Panel A shows the raw signal response for 20, 40, 60, 80, 100, 150, and 200 pM Cy5-labeled anti-E2 antibody. Panel B demonstrates that the slope response is a linear function of the δ and by extension of the antibody concentration (see text) for both Cy5-labeled anti-E2 (●) and Cy5-labeled anti-biotin (■).

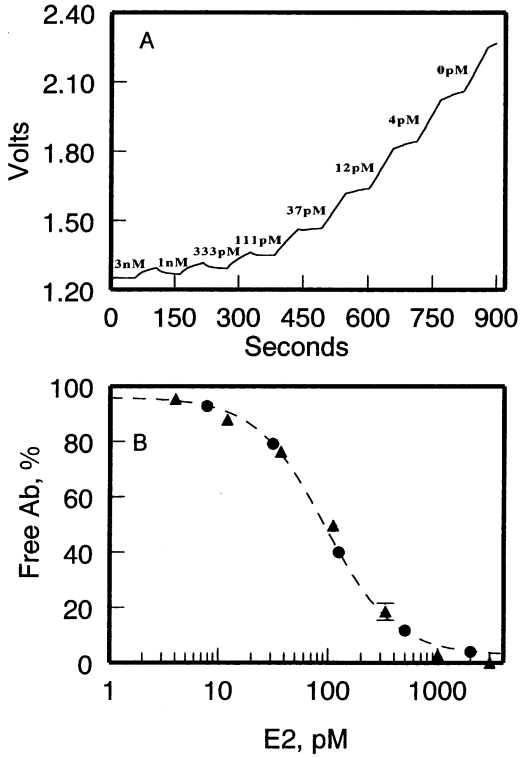

Figure 2. (A) Raw signal response obtained by sequentially passing reaction mixtures containing different concentrations of E2, as indicated in the figure (with a constant Cy5-anti-E2 concentration of 100 pM) over the same solid phase. (B) shows the percent free antibody (extracted from panel A, as described in the text) vs E2 concentration (▲) along with a standard curve generated using end points and separate solid phases for each concentration of E2 (●).

monitoring method. For the assay format to be useful in this regard, two things are necessary: (1) the rate of accumulation of free antibody captured on the beads must be directly proportional to the concentration of free antibody, thus allowing binding slopes to be used as a readout of free antibody concentration, and (2) the solid-phase capacity must be large enough to allow for several consecutive measurements on the same solid phase. The first two experiments presented herein were therefore directed toward confirming the linearity of the binding rate on the beads as a function of the concentration of the free antibody and establishing the feasibility of making several consecutive measurements on a single solid phase.

To investigate the linearity of the relationship between the measured binding slope and the free antibody concentration, 600 μL of an E2-BSA-coated PMMA particle suspension were drawn into the capillary flow cell and allowed to settle. Flow was initiated with 30 s of PBS, immediately followed by 4 min (flow rate, 0.25 mL/min) of Cy5-labeled anti-E2 at varying concentrations. Interstitial solution antibody was removed by a two-stage wash with PBS, first 30 s at 0.25 mL/min and then 1 min at 1.5 mL/min. The results are shown in Figure 1. Panel A shows the instrument response (volts are the response unit of the fluorescent detector and are a measure of fluorescent signal strength) as a function of time, with each trace representing a different concentration of labeled antibody as detailed in the figure legend. A slope was calculated from the linear portion of each trace (between 90 and 210 s) and a δ (difference between the end and beginning of the trace) was also calculated for each trace. The δ is a measure of the free antibody concentration[10] and is used as a control for the slope values. Figure 1B (circles) shows the slope versus δ for the Cy5-anti-E2. As seen clearly in the figure, the binding slope is a linear function of binding δ and, by extension, of the free antibody concentration. These results ensure that the free antibody in the sample can be quantified by measuring the slope of the binding signal. The same series of the experiments was also performed with biotin-BSA-coated PMMA beads and Cy5-anti-biotin, with results shown as squares in Figure 1B.

To check the feasibility of making multiple measurements, varying concentrations (0−3 nM) of E2 were mixed with 100 pM Cy5-anti-E2. An aliquot (500 μL) of each solution was passed sequentially through a single packing of solid phase, resulting in the trace shown in Figure 2A. The percentage of free antibody in each binding mixture was taken as the slope at a given concentration divided by the slope with zero antigen. The nonspecific binding slope (measured at 3 nM antigen, a concentration that ensures that all the antibody binding sites are already occupied with soluble ligand) was subtracted in calculating the percentage free antibody. In a separate series of experiments, the same concentrations of reactants were measured separately using fresh solid phase for each. δ analysis was used, and the percent free antibody was again calculated. The results of both of these experiments are shown in Figure 2B, which demonstrates the complete equivalence of measuring the percent free antibody using slopes measured on the same solid phase or using δ's derived from separate solid phases. The dynamic range of the assay extends from approximately 10 to 1000 pM as shown in Figure 2. From these results, a slope format assay using a single solid phase

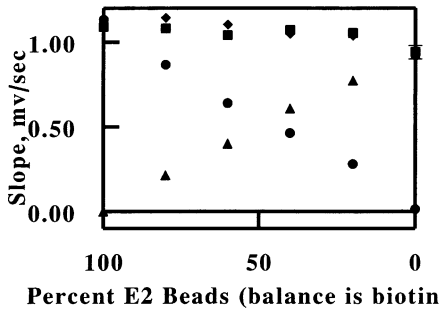

Figure 3. Proportionality of observed binding slopes to the percentage of the solid phase coated with the specific antigen. The Cy5-labeled anti-E2 and anti-biotin antibodies were mixed in a fixed ratio (50 pM anti-E2 and 3 pM anti-biotin), and slopes were measured on bead packs containing the indicated proportions of beads coated with either E2-BSA or biotin-BSA. In control experiments, each antibody was passed individually over bead packs containing the indicated proportions of the two types of coated beads. The data demonstrate that the slopes determined using a mixture of Cy5-anti-E2 and Cy5-anti-biotin (◆) are equal to the sum of the slopes (■) observed for Cy5-anti-E2 (●) and Cy5-anti-biotin (▲) antibody alone, showing the binding events are independent of one another.

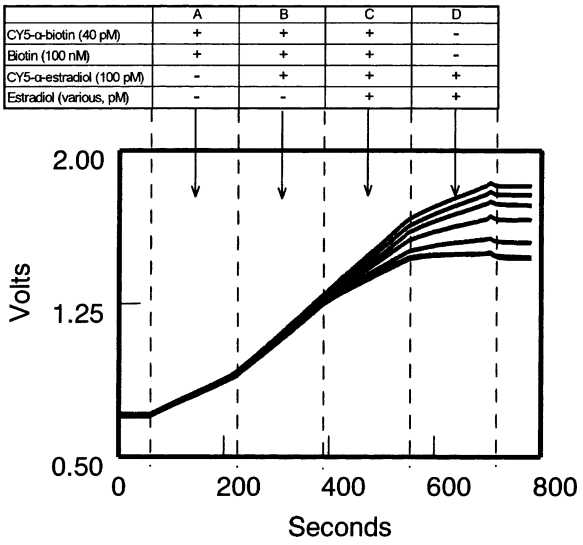

Figure 4. Representative traces for four different mixtures of Cy5-anti-biotin, biotin, Cy5-anti-E2, and E2. The concentrations of E2 represented in regions C and D are 0, 8, 24, 74, 222, 666, and 2000 pM.

is clearly shown to have utility for rapidly making several sequential measurements in a semicontinuous monitoring mode.

In addition to enabling semicontinuous monitoring, combining slopes and multiple measurements on a single solid phase is advantageous from the following three perspectives. First, an entire standard curve was measured in ~15 min as shown in Figure 2. Second, using the same solid phase eliminates one variable when attempting to reference matrix effects in real samples. Third, the excess solid-phase capacity suggested the possibility of coating with two different antigens and using a second antibody as reference for nonspecific matrix effects. These three together set the stage for a reference technique in which known and unknown samples, including both buffers and environmental matrixes, are measured in rapid succession on a single solid phase.

Next, the feasibility of using a second antibody (specific for an antigen not present in the environmental sample) as a reference for matrix-induced nonspecific effects on binding signals was evaluated. The ultimate success of such a scheme depends critically on finding a second antibody exhibiting essentially the same response to the matrix as the detection antibody. As a preliminary to evaluating a pair of antibodies in environmental samples, the capability to independently measure two different antigens on a single (mixed) solid phase was investigated. For the envisioned reference to work, the binding of one antibody must not affect (e.g., through steric hindrance) the binding of the second antibody. To check this, solid-phase materials coated with either biotin-BSA or E2-BSA were mixed in various ratios and used with either Cy5-anti-E2, Cy5-anti-biotin, or a mixture of both antibodies. Results are summarized in Figure 3, which shows the binding slopes measured at different solid-phase mix ratios using each labeled antibody separately and using a mixture of both. As expected, the binding is apparently independent as shown by the slope from the mixed antibodies equaling the sum of the slopes from each antibody applied separately.

Independence of binding was confirmed by measuring an E2 titration curve in the presence of Cy5-anti-biotin. Each measurement included four separate slope determinations. First a mixture of Cy5-anti-biotin and 50 nM biotin was passed over the solid phase, which contained 80% of beads coated with E2-BSA and 20% of beads coated with biotin-BSA. In a second step, Cy5-anti-E2 was added to the mixture, followed by a third step in which various concentrations of E2 were added to the fixed concentration of the labeled anti-E2 antibody. In a final control step, the Cy5-anti-biotin and biotin were removed and the mixture of Cy5-anti-E2 and E2 was flowed alone over the solid phase. Figure 4 shows a family of the curves generated with different concentrations of E2. These data were analyzed by three separate methods: first, the slopes were calculated for the Cy5-anti-E2 and E2 alone (region D in Figure 4); second, the same slopes were calculated by subtracting the slope for Cy5-anti-biotin plus biotin alone (region A in Figure 4) from the slope obtained with these reagents and Cy5-anti-E2 plus E2 (region C in Figure 4). Finally, the data were analyzed by taking the ratio of slopes in region D (Cy5-anti-E2 plus E2) divided by the slopes in region A (Cy5-anti-biotin plus biotin). This last analysis represents the reference method as it is anticipated that referenced matrix effects will cause the same magnitude of changes in both these signals, leaving the ratio unchanged. As shown in Figure 5, these three methods are completely equivalent to each other and to the standard calibration curve included for comparison.

Finally, environmental samples were drawn from a local river and lake (Tone River and Tega Lake). Based on an extensive survey of surface waters,[13] both of these sources are expected to have very low concentrations (<1 pM) of both E2 and biotin. These samples were measured using both the Cy5-anti-biotin and the Cy5-anti-E2 antibodies. Controls of the same reagents added to PBS were run on the same solid phase. Figure 6 is a representative trace of the output, demonstrating the use of a single solid phase to measure 13 separate variations of primary antibody, reference antibody, buffer controls, environmental

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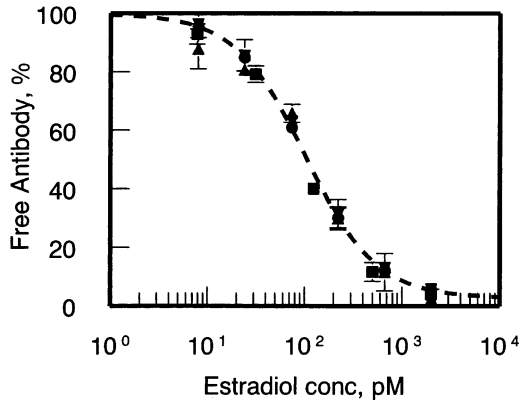

Figure 5. Standard curve calculated from the data of Figure 4, using three different methods of calculation. Circles (●) use the slopes from region D of Figure 4 only. Upright triangles (▲) represent subtracting the slopes from region A from the slopes of region C in Figure 4. Inverted triangles (▼) use the ratio of the slopes from region D divided by the slopes of region A. Squares (■) show results obtained in a separate experiment using fresh solid phase for each measurement and the absence of Cy5-anti-biotin.

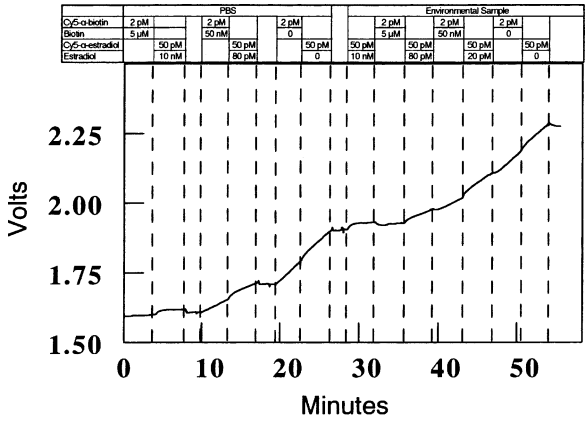

Figure 6. Representative trace from PBS controls and environmental sample (river or lake water, as described in the text) passed over a single pack of mixed beads. The rectangles at the top of the figure show the length of time that various combinations of antibodies and analytes were applied to the same solid phase. A total of 13 separate combinations of matrixes, antibodies, and analytes were applied to the bead pack in this experiment.

samples, etc. The figure is labeled to show what reagents were in the measurement cell at each time point in the experiment. This figure also demonstrates the ability of the system to respond appropriately to high concentrations of analyte after exposure to low concentrations, as well as the reverse. If the reference is to work, the percentage change in binding signal caused by the matrix must be the same for both the Cy5-anti-biotin and the Cy5-anti-E2 binding signals. As shown in Figure 7, this was only partially successful. In the absence of soluble antigen (zero analyte sample) the percentage change in Cy5-anti-E2 signal (environmental sample signal over PBS signal) was the same as the percentage change in Cy5-anti-biotin signal. This result indicated that the matrix was having an equivalent inhibitory effect on each antibody's interaction with the solid phase. This was true for both environmental samples even though the magnitude of the effect was different, with the Tega Lake sample suppressing signals by ~10% while the Tone River suppressed them by ~20%, as shown in Figure 7. However, when soluble antigen was added to each

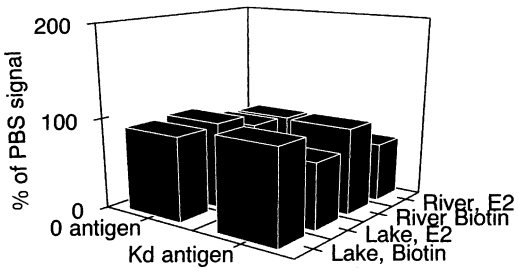

Figure 7. Ratio of signal slope in the environmental sample over the slope in the PBS control (expressed as a percentage) for two environmental sources, with and without added soluble antigen. In the absence of added antigen the Cy5-anti-E2 is suppressed to the same degree as the Cy5-anti-biotin signal. With antigen approximately equal to the K_d, the Cy5-anti-E2 signal is suppressed more than the 0 E2 signal and the Cy5-anti-biotin signal is suppressed less that the 0 biotin signal.

sample at a concentration approximately equal to the K_d for each antibody, the biotin signal was higher than expected in both samples while the E2 signal was lower than expected. The cause for this discrepancy is not presently known. One possible explanation is that the matrix affects the stability of the antibody−antigen complex; i.e., the matrix may induce changes in the antibody that affect its K_d. An alternate explanation may be that unknown compounds in the environmental matrix may interact differently with the two soluble antigens and thus change their apparent affinities for the two antibodies in opposite ways.

CONCLUSIONS

This study established a semicontinuous monitoring method using the binding slope of the free antibody in the sample solution to the solid phase. The established assay relies on an excess solid-phase capacity in which binding of antibody to the immobilized antigen is sufficiently tight and rapid to permit accumulative capture, leading to a quantitative instrument response based on the rate of accumulation. The linear relationship between antibody concentration and rate of accumulation is a result of the constant capture efficiency of the immunoassay system employed. In addition, the solid-phase capacity has been shown to be sufficient to allow multiple sequential slope determinations from a variety of samples up to at least 13 separate samples as shown in Figure 6.

It has been shown previously that the flow-based immunoassay employed here is capable of binding of the free antibody from solution to the immobilized ligand without shifting the equilibrium between antibody and antigen in solution and is thus able to achieve the theoretical sensitivity limit for small antigens.[10] The virtually perfect match between determinations performed on individual solid phases, as used previously[10] and the eight sequential slope determinations, all performed on the same solid phase, suggests that the new assay configuration will maintain the sensitivity of the earlier assay system.

Other workers have used slope as a readout on the same assay system (KinExA) to speed measurement,[14] but we appear to be the first to recognize the utility of the excess solid phase in enabling multiple measurements without interruption for solid-

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phase replacement. Other techniques for continuous measurement such as surface plasmon resonance and quartz microbalance rely on regeneration of the solid phase for sequential measurements, which necessarily interrupts the measurement process. In addition, mass-sensitive techniques such as these may encounter difficulty with environmental samples.

Recently, there has been growing attention on female hormones in sewage treatment plants. Immunoassay is expected to be one of the methods enabling sensitive detection of estrogen. However, there are no assays currently available to quantitatively monitor the hormone in the effluent in real time. In addition, the false positive response in immunoassays of environmental samples has been pointed out as a problem to be solved. This study investigated a possible technique, the use of a second antibody as a reference, to avoid the false positive responses in the direct measurements of environmental samples. The preliminary results presented herein show there is promise in this approach for eliminating a false positive response for the analyte. Additional experiments are underway to identify and specifically select antibody pairs that are sufficiently similar in their response to the matrix to allow completely successful referencing.

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