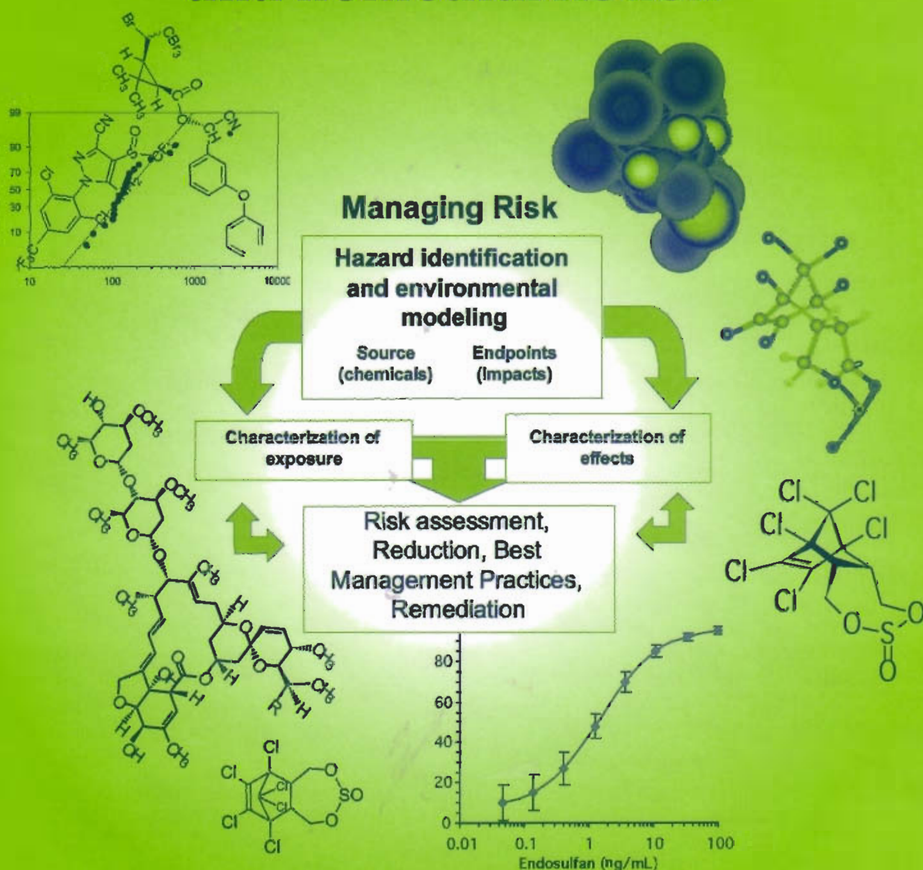
ACS SYMPOSIUM SERIES 966

Rational Environmental Management of Agrochemicals

Risk Assessment, Monitoring, and Remedial Action



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Monitoring

Chapter 9

Progress in the Development of Biosensors for Environmental and Human Monitoring of Agrochemicals

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Monitoring studies for toxic substances in environmental and biological samples are essential for evaluating risk to human and environmental health. Immunoassays are one method that can satisfy analytical demands for high sample loads requiring high sensitivity because they are rapid, can be adapted to high throughput or biosensor formats and can be adapted for small sample sizes. A description of progress in the field of immunoassays and the application to biosensors in general is presented with a focus on research from this laboratory.

In order to make rational decisions on the selection and use of agrochemicals, data from environmental fate and target and non-target organism exposure as well as human exposure are needed, in addition to continuing monitoring studies to validate these decisions. Environmental monitoring studies of the residues of toxic chemical contaminants can show how residues influx to humans from environmental media such as air, water, food, consumer products, soil and settled dust. These studies answer questions about source for exposure (anthropogenic/non-anthropogenic, area/point, and indoor/outdoor); the transport/carrier medium; the exposure pathway (eating, breathing, touching); and how long the residues exist *(1)*. In evaluating human exposure,

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biomonitoring studies assess the nature of the health hazard; how many people are exposed; to how much; identify the exposure pathway (inhalation, ingestion, or dermal contact); determine the residual concentrations in people; define the relationship to human health effects; and identify special populations or individuals that may be exposed.

These two types of studies help provide the most complete exposure information for toxicologists and epidemiologists to evaluate the potential for adverse health effects on humans or other non-target organisms and the environment. The data can be used to select strategies to maximize crop production with proper planning and implementation of pesticide/pest management practices while minimizing adverse exposures to humans and the environment. Such complex monitoring studies require hundreds or thousands of samples to obtain sufficient data. Thus, there is a need for rapid, quantitative, high throughput analysis methods.

Gas chromatography (GC) or high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) are the first choice of methods for large scale screening studies for multiple analytes for both biomonitoring *(2, 3)* and environmental monitoring *(4)*. These techniques are well documented and give accurate results. Along with instrumental methods, affinity-based analyses can play an important role in high-throughput applications. Such affinity-based assays rely on biological reagents such as enzymes, DNA/RNA aptamers and antibodies or chemical reagents such as molecularly imprinted polymers.

Antibody-based immunoassays have been proven to be a rapid, sensitive and cost effective analytical tool for routine monitoring *(5)*. They are particularly well-suited to analysis of certain substances that are more difficult to analyze with GC or HPLC because of large molecular mass, thermal lability, low volatility or lack of a distinct chromophore. Antibodies in immunoassays act as a receptor (detector) for the analyte of interest. Binding occurs through hydrogen bonding, hydrophobic bonding, electrostatic, van der Waals forces and by the degree of complementarity between the antibody and the antigen, making it an ideal method for compounds with physical and chemical properties that are difficult to analyze by other instruments *(6, 7)*.

Moreover, immunoassays can be formatted to use only a small volume of sample, are adaptable to high throughput methods using the 96- or 384-microwell plate or current autoanalyzer systems and are particularly useful as the primary screening method to reduce a large sample set by avoiding the need to completely work-up negative samples. In this scheme, conventional instrumental analyses with GC and/or HPLC that can then focus on the more interesting and positive samples *(8)*. But immunoassays can also be used as the primary means of analysis as exemplified by a study carried out in our laboratory in which paraquat was measured in urine collected from farm workers exposed to the herbicide in order to study the relationship of exposure to agricultural lung disease *(9, 10)*. The paraquat immunoassay combined with a cation exchange solid phase extraction (SPE) method for urine extraction was demonstrated as an

excellent tool with the advantages described above *(11)*. Immunoassay methods are accepted by regulatory agencies as demonstrated by the validated method for the natural insecticide spinosad in ruminant commodities that is set for inclusion in the US Food and Drug Administration Pesticide Analytical Manual Volume II *(12)* as well as methods described in the Environmental Protection Agency Residue Analytical Methods *(13)* and those submitted to the EPA Office of Pesticide Programs as part of registration packages.

As an improved tool, biosensors result from the association of a sensitive biological element with a transducer, which converts the biological signal into a measurable physical signal (Figure 1). The biological element may be an enzyme, antibody, cell, DNA, DNA/RNA aptamer or receptor.

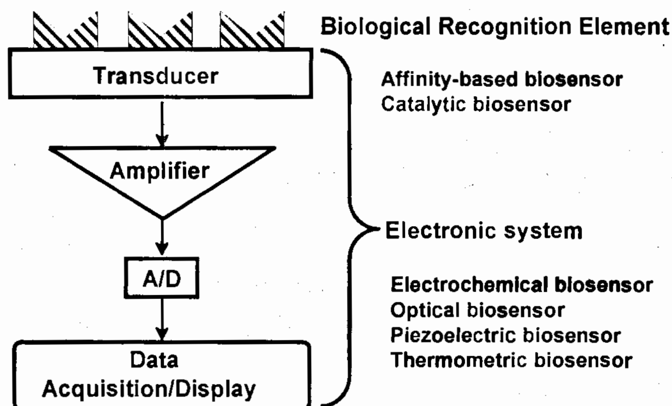

Figure 1. Schematic of a biosensor

A recent review describes the applications of these various types of elements in biosensors for environmental monitoring *(14)*. The transducer is in close proximity or is integrated with an analyte-selective interface *(15)*. The transducer signal may be mass (microbalance), electrochemical or optical (surface plasmon resonance, fluorescence, chemiluminescence). Descriptions of types of signal transduction for biosensors is given in Suri *et al.* *(16)*. Immunosensors are a specialized type of biosensor, which utilize antibodies for detection. Antibody-based sensors can provide continuous, *in situ*, rapid and sensitive measurement based on the conventional immunoassay.

To develop an integrated affinity-based biosensor requires research into the components of the immunoassay (haptens, binding molecules, formats, labels) as well as the the detection system, the reagent delivery system, etc. Each of these alone is a large area of research. We review briefly here advances in selected areas of biosensor research for agrochemicals.

Assay Improvements

Immunoassays for small molecule environmental toxicants, such as pesticides, are developed with the processes of synthesis of haptens as immunogens and coating antigens. Using the immunogens, antibodies are produced. The antibodies are screened against a library of competitor haptens (coating antigens). Assays with high sensitivity and selectivity are optimized to avoid interferences and perform in the matrix of interest and finally the assay is validated with real analytical samples. An overview of this procedure is described in Suri *et al. (16)*.

Hapten design approaches

Since small molecules, like environmental toxicants, are not immunogenic, mimics of the toxicant that contain functional groups for use in coupling to carrier molecules (e.g. peptides, protein, polymers) are synthesized, coupled and used to immunize animals to generate antibodies. For immunizing a close structural mimic of the toxicant of interest is required (immunizing hapten). In analyzing the toxicant, an immunoassay is usually competitive. That is, the toxicant (analyte) competes with an analyte mimic coupled to a carrier molecule that is used in the assay (competitive hapten). The more effectively the analyte can compete, the lower the detection limit of the assay. Many assays use the same hapten for immunizing as for competition in the assay. The drawback is that the antibody may have a higher affinity for the analyte mimic, than for the free analyte. One strategy to overcome this drawback, is to synthesize other mimics of the analyte (heterologous haptens) that will bind with less affinity to the antibody. One approach is to change the bridge chemistry (e.g. handle length or coupling chemistry). For example with the herbicide atrazine, changing the bridge length from two carbons to six carbons resulted in a more sensitive assay *(17, 18)*. One can also replace part of the structure (e.g. with an isoster, S/Cl or to substitute another atom with less hydrogen binding capacity such as a carbon for a nitrogen) or to use a partial structure *(17)*. This strategy was used successfully for the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Substituting phage-displayed peptides as mimics of the hapten is a recent approach. The phage display system produces randomly generated amino acid sequences fused to coat proteins of the filamentous phage *(19, 20)*. By utilizing the phage display method, a vast repertoire of peptides can be expressed as libraries. The phage libraries can be rapidly screened by enriching for specific clones by repetitive rounds of affinity selection. The peptides are typically 12-16 amino acids and can be selected with a high affinity *(21)*. Use of peptide haptens in assays for molinate and atrazine resulted in detection limits that were similar (for molinate) or lower (atrazine) than the assays with the corresponding chemical hapten *(22)*.

Improved binding molecules

Improvements to the sensitivity, selectivity and/or ruggedness of the assay have also been approached by using modified antibodies (antibody fragments, recombinant antibodies, sol gel-encapsulated antibodies), biological antibody replacements (peptides, aptamers) or chemical replacements (molecularly imprinted polymers). The most desirable antibodies for small molecule analysis have a high affinity for the analyte of interest, are stable under the conditions of analysis, stable to potential matrix interferences, exhibit low background in the assay and have limited cross reactivity to non-analyte molecules. Antibodies are immunoglobulins that consist of four fragments (two identical light chains and two identical heavy chains). The fragment of antibodies that bind antigens (F_{ab}) consists of variable regions of the light and heavy chains linked by a disulfide bridge through a constant region associated with each chain. F_{ab} fragments are produced by enzymatic cleavage of the antibody molecule. F_{ab} fragments are sometimes desirable as their use produces lower background in some formats, but the fragments often lack the avidity of the whole molecule *(23)*.

Using recombinant techniques, the variable regions of the light and heavy chains can be synthesized (without the associated constant region) and their respective N- and C-termini linked by a peptide bridge. These antibodies are termed single chain fragments of the variable region (scFv). Advantages to recombinant technology are the ability to systematically improve the primary binding site and to create fusion proteins. Fusion proteins contain the primary antibody protein fused to a reporter protein. Such a fusion protein has been made for the detection of atrazine using the enzyme alkaline phosphatase *(24)* and for picloram using a fluorescent protein *(25)*. Fusion proteins that facilitate separation in a homogeneous assay for atrazine have also been reported *(26)*. Reviews of the development of the field of recombinant antibodies for environmental analysis is presented by Kramer and Hock *(7)* and Yau *et al. (27)*. Application of assays using recombinant reagents for pesticide environmental analysis have been reported for atrazine *(7)* and simetryn *(28)*.

Similar to hapten strategies described above, phage displayed peptides can also mimic the antibody. To our knowledge there are no reports utilizing phage display peptides as binding molecules for small molecules for environmental analysis. However, there are a large number of reports for phage display binding to peptides and larger antigens such as cell surface antigens or receptors. Further details of this technology and uses for peptide binding molecules can be found in a review by Kehoe and Kay *(29)*. For most environmental immunoassays, the analyses are in a competitive format, rather than a sandwich format. One novel use for peptides would be to detect a small molecule bound to it's capture antibody. In this way the assay would more closely resemble a sandwich assay and would be a way to more easily utilize array technologies *(30)*.

Nucleic acids are typically used as probes to hybridize with and detect RNA and DNA target sequences that are complementary. Recently, aptamers, short

synthetic DNA and RNA sequences, are being used as ligands to bind to targets other than nucleic acids with high affinity and specificity. Although typically used to detect peptides and proteins, aptamers have been developed to detect compounds as small as ethanolamine *(31)*. They are especially attractive as analytical reagents because they are more temperature stable and more easily produced than antibodies. On the other hand, their binding ability depends on their folding and three dimensional structure which is susceptible to incubation and buffer compositions *(32)*. Aptamers are commonly 'synthesized' via a combinatorial chemistry technique known as systematic evolution of ligands by exponential enrichment *(33)*. Resulting aptamers have application in affinity separations, as the binding element in biosensors or as signaling molecules (i.e. molecular beacons) *(34)*. A biotinylated RNA aptamer made against L-2-phenoxypropionic acid was bound to streptavidin-coated silica to make a solid support for capillary electrophoresis. The capillary column proved robust in that more than 700 injections at concentrations of acetonitrile of 5-30% and temperatures of 5-40 °C showed no differences in retention time and peak shape for several herbicides in the aryloxyphenoxypropionic family *(35)*. In addition, the column was capable of separating the enantiomers of the herbicides. Recently, aptamers for cocaine have been applied to a label-free biosensor. One end of the aptamer is immobilized on a gold electrode surface. The other end is tagged with methylene blue. When a cocaine molecule is bound by the aptamer, the methylene blue-tagged end comes in proximity to the electrode surface generating an electric signal. With the ease of making aptamers, this system should be generally applicable to detection of pesticides in environmental or biological samples *(36)*.

Improved labels

Classically, labels to detect antibody binding have been radiometric, colorimetric, or fluorescent/chemiluminescent. Labels that can be detected sensitively, with high signal:noise ratio, that are stable and robust are ideal. Fluorescent labels are generally thought to be detected with greater sensitivity than colorimetric labels, but they can subject to interference from background fluorescing materials, photobleaching, short half lives and narrow Stoke's shifts. An improvement would be to use labels that fluoresce at longer emission wavelengths and/or chemiluminescent materials. Detection of such labels would be away from wavelengths where naturally fluorescing materials occur, thus reducing background interference. We have focused on the lanthanide europium oxide (Eu_2O_3) nanoparticles as fluorescent labels. There is a large body of literature using the europium ion as a reporter for immunoassay when complexed in a chelate, but few reports using the lanthanide alone. The lanthanides have large Stoke's shifts, sharp emission peaks, emission at a wavelength generally free of interference from natural biological fluorescence and a long half-life to

facilitate the use of time resolved mode, further increasing the signal to noise ratio.

In one example of our work, inorganic Eu_2O_3 nanoparticles were used as a novel fluorescent reporter in an immunoassay for 3-phenoxybenzoic acid (3-PBA) a breakdown product of a number of pyrethroid insecticides *(Ahn et al., unpublished data)*. The surface of the particles was functionalized with either an aminopropyltrimethylsilane using a microwave coating method or with a simple aminopropylsilatrane (APS) coating method under aqueous conditions. As shown in Figure 2, NH_2-functionalized particles were coupled to 3-PBA for a competitive immunoassay.

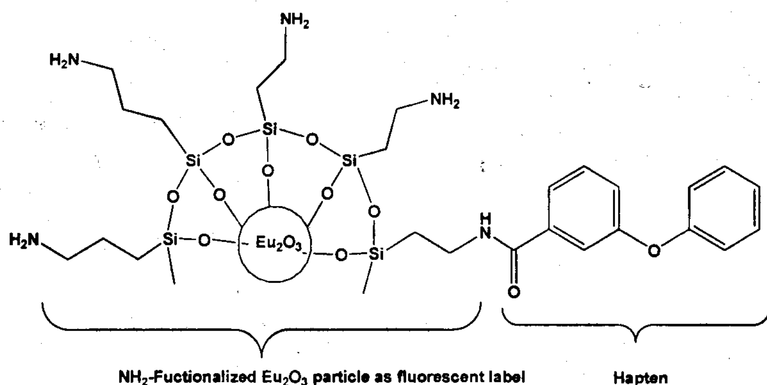

Figure 2. Putative structure of europium particle-labeled hapten

The Eu_2O_3-fluorescent immunoassay using a magnetic separation technique and paramagnetic secondary antibody in the assay procedure remarkably improved the sensitivity, compared to the conventional microplate ELISA (*37*). However, an assay for 3-PBA-glycine using particles coated by the microwave method was not as sensitive as the conventional microplate ELISA (*38*). This suggests that the coupling technique is important to the performance of the assay, but further research is necessary. The fluorescence immunoassay is simple and rapid, required no washing steps and no enzyme conjugates, making it of significant utility for a high-throughput assay.

Improved Throughput Techniques

Immunoaffinity Chromatography

One approach to improving throughput for agrochemical analysis is to adapt immunoassays to combine cleanup and detection into one device. An immunoaffinity chromatography (IAC) is based on a solid-phase extraction principle, but utilizes the selectivity of antibody to isolate and concentrate a

specific antigenic analyte from a sample matrix. Immunoaffinity matrices are developed by chemically coupling the ligand (a pure antigen, an antibody or an anti-antibody) to a suitable matrix such as sepharose or agarose. An IAC generally enhances the detection sensitivity, recovery rate and analytical time. The resulting extracts for the subsequent detection is generally cleaner than those of the conventional solid-phase extraction methods. They consist of less interfering substances leading to less interfering peaks in the chromatographic detection, hence improving the detection sensitivity.

Coupling immunoaffinity chromatography to an instrumental system allows a fully automated system. This design combines the advantages of high selectivity and throughput capacity of immunoaffinity chromatography with that of high accuracy/precision of the instruments. Some successful examples include analysis for β-agonists *(39)*, benzene/toluene/ethyl benzene/xylene *(40)*, isoproturon and fluoroquinolones *(41)*.

A disadvantage is that the commercial immunoaffinity columns only warrant a single use, although re-generation for repeated usage may be possible *(42)*. Reusability is dependent upon various factors including the type of antibody, specific antibody-antigen interaction involved and elution conditions being used. Immunoaffinity chromatography has been re-used as many as ten times without significant loss of performance.

One way to stabilize antibodies to make them more reusable in IAC applications is to encapsulate them in a matrix such as a sol-gel. Sol-gel encapsulated antibodies retain their binding capacity and are being widely investigated for application in biosensor development *(43)*. For pesticides, sol-gel-based immunoabsorbents have been used for the detection of the herbicide isoproturon *(44)*, triazine herbicides and the insecticide malathion *(45)*, and sulfonylurea herbicides *(46)*.

Unlike immunoassay, an IAC requires a fairly large amount of antibody to be effective - at least 0.1-1 mg per column. Thus monoclonal antibody is the current general source of antibody if a bulk production is intended. However, as mentioned above, other binding reagents such as recombinant antibodies, phage displayed peptides or aptamers could be used. Like immunoassays, immunoaffinity columns perform best within their expiration dates and their shelf life is relatively short (up to one year in most cases). Because they are still produced in batches, there may potentially be some batch-to-batch variation, which may require an additional validation of every new batch being produced.

IAC application in the area of food contaminants other than pesticide residues has been active and a number of IAC are now commercially available for this purpose. Environmental application of IAC has been slow possibly due to skepticism within the general analytical community. Also the availability of effective sample preparation methods for environmental residues could have impeded the development of IAC for environmental application. However, advances in antibody stabilization and miniaturization, make the IAC approach attractive for biosensor development.

Immunochromatography

Immunochromatography combines the power of immunochemistry and chromatography on a membrane. Membrane or test strip based assays can exist in various forms such as dipstick, lateral flow device and vertical flow device. These devices are designed to augment the performance of immunoassays in a more user-friendly fashion, delivering results in minutes with either no or very simple extraction. The greatly enhanced portability in ready to use test kits has made this technology very attractive as a decision support tool to industries and regulators in recent years. The advantages of such rapid systems are ease of handling, no washing procedures, no substrate required, results can be obtained within minutes and results are permanent (when visualization system other than enzyme-substrate system is used). However, the main drawback of the current rapid systems is low sensitivity compared to their alternative formats.

A dipstick assay with an immobilized antibody works on the same principle as in a competitive immunoassay of a microwell plate, a membrane strip is first spotted with a hapten-specific antibody and a label-specific antibody such as an anti-enzyme antibody. The hapten-specific antibody must be able to interact with the sample analyte and the competitor hapten within a short contact time. The tracer-specific antibody, on the other hand, must be capable of binding with the tracer to serve as a control or a reference *(47-50)*. When performing the assay, the strip is immersed into a sample solution containing a known quantity of labelled competitor/tracer to allow competition between the analyte and competitor to occur. When no analyte is present, maximum color would develop. When the analyte is in excess of the labelled competitors, the test dot will remain invisible when the substrate is added. A dipstick assay with immobilised antigen can be performed with a similar approach except that the membrane will be immobilized with a hapten competitor (hapten-protein conjugate) and an anti-species antibody (as a control). The system would be visualized by a labelled hapten-specific antibody coupled to an enzyme. The dipstick assays have been developed for carbaryl *(51)*, atrazine *(52)*, terbuthylazine *(53)*, ametryn *(54)*, fenthion *(50)* and 2,4-dichlorophenoxyacetic acid *(55)*.

Unlike a dipstick, a lateral flow device involves the transport of sample analyte and labelled-antibody through a test strip to the testing zones by capillary action. The immobilization is generally achieved by striping to obtain fine lines (generally 1mm thick). During the transport through the membrane, the sample analyte is given sufficient time to compete with the labelled protein, while larger unwanted molecules are hindered by the small membrane pores. Such a separation mechanism helps to reduce interference to the required immunoreactions at the upper part of the test strip, thereby improving the performance compared to a dipstick *(56, 57)*. The adsorbent pad on the lateral

flow device, where the sample is applied, also acts like a filter and further helps to reduce interference moving with the antibody. The lateral flow or flow through devices have been developed for 2,4-dichlorophenoxyacetic acid *(58)*, alachlor *(56)*, carbaryl *(57)* and endosulfan *(57)*.

A typical vertical flow device consists of a plexiglass base, an absorbent layer, membrane coated with antiserum and a plexiglass lid with holes for introducing samples *(59-63)*. Membrane pieces (typically of 3 x 3 cm^2) are firstly striped with hapten-specific antibody and tracer-specific antibody. Upon blocking, the membranes are fixed into the test device. Standard or sample solution, wash solution, labelled competitor hapten solution and wash solution are then dropped sequentially onto the membrane with a 1-1.5 min interval between each solution. This permits the liquid to be adequately absorbed by the membrane and interact with the immobilized components. Any excess or unwanted components will be absorbed by the absorbent layer underneath.

When an enzyme is used as a tracer, substrate solution will then be added for color development. The incubation time for this process is approximately one minute *(60)*. The reaction is subsequently stopped by washing with a surfactant such as Tween 20. The total test time for this assay is typically around 10 min *(60, 62)*. The absorbance of colour *(63)* or fluorescent complex *(61)*an be directly measured by a reflectometer. If colloidal dye, colloidal gold or luminal particles are used in place of an enzyme-substrate system, imaging system such as FluoroImager can be used for visualization and subsequent quantification. Assay time with such visualisation systems can be as short as 2 min. The immobilization of antibody, flow techniques and rapid visualization lend itself the to development to the design of lab-on-a-chip type biosensors.

High throughput platforms

Another approach to improving throughput is to adapt existing environmental immunoassays to current clinical automated, high throughput platforms. We have adapted a competitive chemiluminescent assay for the detection of 3-PBA based on polyclonal antibodies with an automatic Bayer ACS:180 immunoassay analyzer system. The chemiluminescent acridinium ester label was linked to the 3-PBA-BSA conjugate. The optimized competitive immunoassay format showed high sensitivity (IC_{50} values of 0.3 µg/L) for 3-PBA, compared to that (IC_{50}=2 µg/L) of the conventional microplate ELISA. In this method, the competition step is performed offline, then the immunoassay was performed by the fully automatic analyzer using paramagnetic secondary antibody for the separation of immunocomplex and non-immunocomplex. A mixed-mode solid phase extraction (C8 and anion exchange) used to prepare samples prior to analysis to reduce matrix interferences increased the assay

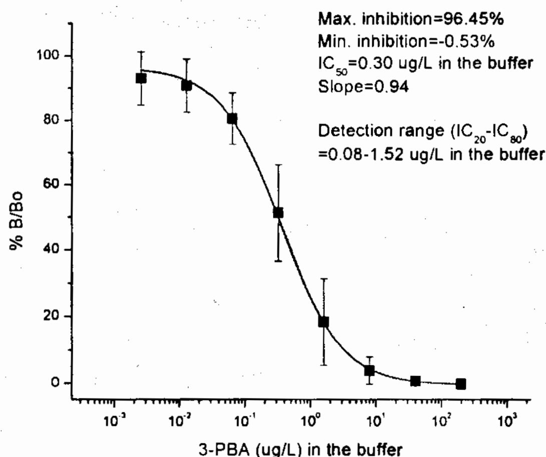

Figure 3. Calibration curve to 3-PBA in the ACS:180 chemiluminescent assay format.

sensitivity, resulting in measurement as low as 1 μg/L urine of 3-PBA with a limit of detection (LOD) of 0.08 μg/L in buffer (Figure 3).

The assay on the ACS:180 provided the first results within 15 min, and yielded around 130 results per hour. The analyzer combined with the highly sensitive chemiluminescent acridinium label, magnetic particle separation, and the automatic processes will support high throughput assay for monitoring human exposure to pyrethroids by measuring 3-PBA *(Ahn et al., unpublished data)* and can easily be applied to analysis of other pesticides using reagents already developed for the classical enzyme linked immunosorbent assay. The automated chemiluminescent immunoassay has excellent advantages in terms of sensitivity, rapidity, and simplicity for application to monitoring studies.

Multiplex fluorescence bead-based immunoassay technology has recently been introduced as a high throughput method for multianalyte screening. When particles are used, the speed of an assay compared to a microtiter-well plate assay can be accelerated because of the mobile property and the larger surface area *(64)*. The small size of polymeric and inorganic particles emitting light or a sensing element homogenously distributed throughout the particle can be used as label markers, and satisfy criteria for high brightness, a unique signal, and relatively low cost *(64)*. The microparticles encoded with different amounts of a dye can be distinguished with the flow cytometric assay *(65)*. The particle assay can be convenient to separate the free and bound complex. It can efficiently reduce interference caused from sample matrices by separation with filtration or magnetism when the antibodies are attached to paramagnetic particles. Unlike the ACS platform that is one sample, one analyte, the mutiplex bead assay is one sample, multiple analyte format. The fluorescent flow cytometric assays

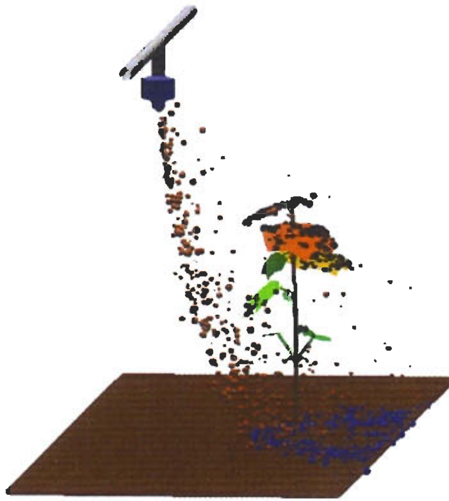

Figure 4.1. Simulation of the movement of spray droplets and deposition on a cotton plant

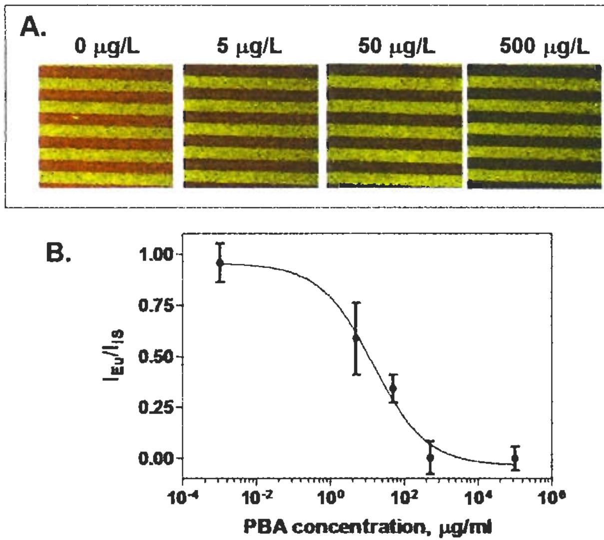

Figure 9.4. Microimmunoassay for 3-PBA. (A) Fluorescence images of glass substrates incubated with different concentrations of 3-PBA. (B) Standard curve for the PBA microimmunoassay. (with permission from Nichkova, 2005)

combined with the use of different sized polystyrene microbeads allows one to perform specific and quantitative immunoassays. This technique was demonstrated for the detection of target analytes, glyphosate, atrazine and the mercapturate of metolachlor *(66)* in urine of exposed farmworkers. The least detectable dose of glyphosate, atrazine and metolachlor mercapturate in urine were 0.9, 0.7 and 0.3 ng/mL respectively similar to or lower than determined by HPLC. It is possible to expand this technique up to 100 analytes according to the manufacturer, but one must keep in mind that cross-reacting analytes can complicate the interpretation of results. One advantage of this method is that it is currently used to detect biomarkers of effect such as cytokines as indicators of inflammation. It is a simple step to combine reagents for the detection of pesticide metabolites as biomarkers of exposure and from one sample have data to help in the assessment of the continuum from exposure to effect in humans.

Microarrays

Protein microarrays have the potential to play a fundamental role in the miniaturization of biosensors, high throughput drug screening, clinical immunological assays, and protein-protein interaction studies *(30, 67)*. A quantititative microarray for atrazine, and the metabolite of dichlobenil, 2,6-dichlorobenzamide has been reported. The microarray was constructed using a spotter to place hapten conjugates in discrete spots on a glass slide. The slide was then subjected to UV light to covalently immobilize the conjugate. A fixed abount of Cy5-labeled antibody for each analyte was added along with the sample or standard. The lowest detection limits for 2,5-dichlorobenzamide and atrazine were 1 and 3 ng/L, respectively *(68)*. As in a typical ELISA, the free analyte competed with the immobilized conjugate for a fixed number of antibody binding sites. After a wash step, bound antibodies are visualized by confocal microscopy. Like many microarrays, the one described here was visualized and analyzed using organic fluorescent dyes *(69)*.

To overcome photobleaching and spectral overlaps we applied a new type of fluorophore, crystalline europium-doped gadolinium oxide ($Eu:Gd_2O_3$) nanoparticles, as labels in protein and antibody microarrays *(70, 71)*. The $Eu:Gd_2O_3$ nanoparticles were synthesized by spray pyrolysis and they offer narrow red emission, large Stokes shift, photostable laser-induced fluorescence with a long lifetime (1 msec) *(72)*. Recently, the nanoparticles were successfully applied as reporters in a competitive fluorescence microimmunoassay for 3-PBA *(73)*. The nanoparticles were functionalized with reactive amino groups by poly-L-lysine encapsulation The PL-encapsulated particles were covalently conjugated to the specific anti-PBA antibody. The microarrays were fabricated by direct microcontact printing *(69)* of the coating antigen BSA-PBA in line patterns (10 x 10 μm) on glass substrates. The nonprinted surface was blocked with BSA-fluorescein.

The competitive immunoassay for PBA was carried out with a preincubation of the labeled antibody (anti-PBA IgG-PL-Eu:Gd_2O_3) with the analyte. Then the glass substrates (printed BSA-PBA/blocked BSA-fluorescein) were incubated with these solutions for 1h where the non-saturated binding sites of the labeled antibody bind to the printed PBA. Confocal fluorescence microscopy combined with internal standard (fluorescein) calibration was used for quantitative measurements. Representative fluorescence images of the patterns obtained for the PBA concentrations tested are shown in Figure 4a. The yellow strips correspond to the internal standard (fluorescein) fluorescence which has similar intensity for the four substrates. Increasing PBA concentration leads to smaller amounts of nanoparticles bound on the printed BSA-PBA strips and therefore to a decrease in the red Eu:Gd_2O_3 fluorescence intensity. The resulting dependence of the normalized specific fluorescence signal (I_{Eu}/I_{IS}) *versus* the PBA concentration is presented in Figure 4b. The parameters of the sigmoidal fit of the competitive immunoassay are: IC_{50} = 14 µg L^{-1}, slope = -0.6, R^2 = 0.987. The detection limit defined as 20% inhibition corresponds to 1.4 µg L^{-1} PBA which is comparable with the reported ELISA for the same analyte *(37)*. This work suggests the potential application of lanthanide oxide nanoparticles as fluorescent probes in microarray and biosensor technology, immunodiagnostics and high-throughput screening.

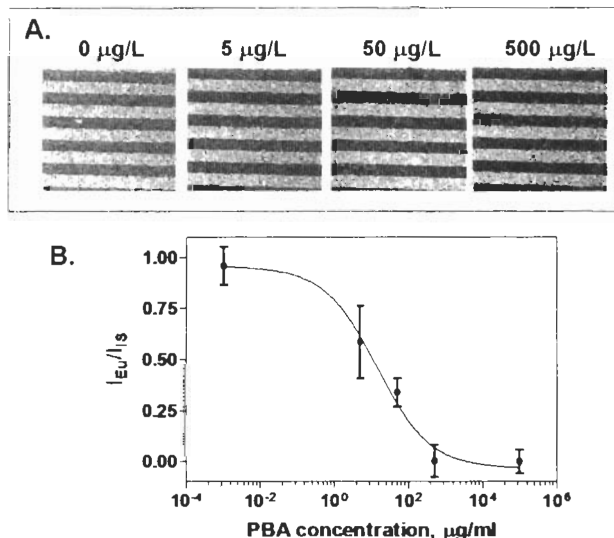

Figure 4. Microimmunoassay for 3-PBA. (A) Fluorescence images of glass substrates incubated with different concentrations of 3-PBA. (B) Standard curve for the PBA microimmunoassay. (with permission from Nichkova, 2005) *(See page 1 of color inserts.)*

Outlook

Our laboratory continues to explore new immunoassay approaches such as a non-labeled immunosensor based on ellipsometry *(74)*. Ellipsometry measures the molecular density of thin films. When the antibodies leave the surface, ellipsometry detects the change in molecular density. Larger antibodies or antibody complexed with other proteins could be used to increase the signal.

The optical properties of colloidal crystals provide a method of transducing biological binding to optical signals. Optical signals are fast and easily used in electronic devices. When assembled, these colloids refract light at a certain wavelength. When the crystals with an antibody are coated on the substrate, the refracted light will change wavelength when the ligand binds to the antibody. The wavelength shifts are based on changes in size, charge and shape of the colloids *(75)*,

Although there are many demonstrations of antibody-based biosensor technology, there are few examples of application to real world environmental samples for pesticides *(76)*. New approaches as well applying what we have learned to develop new systems such as a simultaneous multiplex assay using the flow cytometric system with inorganic lanthanide labels instead of the classic organic fluorophores we hope will lead to systems well-suited for human biomonitoring as well as environmental monitoring, making immunoassays even more valuable tools.

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