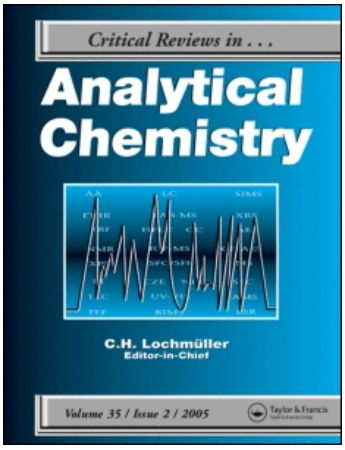

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A Comprehensive Overview on the Application of Flow Injection Techniques in Immunoanalysis

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A Comprehensive Overview on the Application of Flow Injection Techniques in Immunoanalysis

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ABSTRACT: For more than a decade, immunoassay methods have held great promise for use not only in their classic clinical applications but also in food, pesticides, process and quality control, industrial fermentations, etc. A great number of publications have appeared in these fields.

Flow injection analysis (FIA) is a technique that allows the automatic analysis of a large number of samples and/or continuous monitoring, and it has been proven to be applicable to a variety of analytical problems. Nevertheless, antibody-supported flow injection systems have received relatively little attention. Only recently, the advantage of the specificity inherent to most immune reactions began to be exploited in FIA procedures. Flow injection coupled to immunoassays (FIIA) is arising as a powerful tool for the development of analytical protocols, and the application of FIA techniques is expected to contribute enormously in improving the speed and quality of immunoassays. This article gives an overview on the applications of flow injection techniques in immunoanalysis as well as examples of various immunoanalysis formats commonly employed to visualize the primary antibody-antigen reaction. Finally, recent developments and new trends in the field of FIIA, such as immunosensors, are also considered.

KEY WORDS: flow injection analysis, immunoassay, review.

I. INTRODUCTION

Immunoassays can be defined as analytical methods that are based on the detection and measurement of the antigen-antibody reaction. Although the term *immunoassay* often refers to a quantitative method, in a broader sense it also includes characterization methods to analyze the immunological properties of analytes. Two essential elements of an immunoassay system are the reagents and the format. Reagents include the antibodies, antigens, and other chemicals used to visualize the primary binding reaction. The format refers to the device or system into which the reagents are placed to perform the assay.

The first immunoanalytical techniques were based on immunoprecipitation: insoluble antigen-antibody complexes are formed when both species react in appropriate concentrations. However, such techniques can be considered only as semi-quantitative and are not very useful when high sensitivity and accuracy are required. Radioimmunoassays (RIA), based on the isotopic labeling of the antigen or the antibody, constituted a great advance in the field of quantitative immunoassays, taking advantage of the high sensitivity inherent to the radioactivity-based measurements. The next important advance in immunoassays was the development of the enzyme immunoassay (EIA). The first EIA techniques were independently described in

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1971.[1,2] Since then, EIA techniques have been used to detect a wide variety of different antigens and antibodies. The principles and procedures of EIAs are basically similar to those of RIA techniques, except that instead of using a radiolabel, an enzyme label is used, its activity being measured by its action upon a specific substrate.

EIAs are based on two important biological facts: first, the enormous discriminatory characteristics of antibodies, based on the ability of the immune system of vertebrates to virtually produce an unlimited variety of antibodies, each with an affinity for a specific foreign compound (antigen or hapten); second, the high catalytic power and specificity of enzymes that make them easily detectable. EIAs thus consist of two main steps: the primary reaction between the immunoreactants (antibody and its corresponding antigens) and the detection using enzymes previously bound to the reactants as indicators. EIA techniques can be divided into two main groups: enzyme-multiplied immunoassay techniques (EMIT) and enzyme-linked immunosorbent assays (ELISA). In EMIT, the immunological reaction occurs in a homogeneous liquid medium, and separation between bound and unbound reactants is not accomplished. In ELISA, part of the reactions occurs on a coated solid phase (normally a polystyrene matrix) that also serves to separate immunocomplexes from unbound reactants.

ELISA methods can be performed using competitive and noncompetitive formats.[3] In a typical competitive ELISA, the analyte occurs in variable concentrations in the reaction mixture in contact with the specific antibody, and competes with a constant amount of analyte previously immobilized onto the solid phase. The antibody concentration must be limited in order to ensure the effectiveness of the competition. In a noncompetitive assay, the antibody reacts proportionally with the amount of analyte in the sample. Often, noncompetitive ELISAs are performed in a sandwich format, in which the analyte is entrapped between two layers of different specific antibodies, one of them attached to the solid phase and the other one free in solution. In both methods, the amount of antibody finally bound to the solid phase is proportional (inversely or directly) to the amount of analyte in the original sample. This bound antibody can be detected in turn by two methods: direct or indirect. In the direct method, the antibody has been linked previously to an enzyme, whereas in the indirect method a new enzyme-linked antibody is used to specifically recognize and bind the former.

At present, EIA techniques have almost replaced RIA because they avoid the unpleasant use of radioactive isotopes. In addition, a well-designed ELISA is able to reach similar sensitivity and accuracy to those offered by RIA. In recent years some variations of EIA have been described, among them one of the most promising seems to be the fluorescent immunoassay techniques. The principles are the same as those of EIA techniques, but the labeling is a fluorescent marker, which in most cases provides enhanced sensitivity and shorter reaction times. Since their appearance, immunoassays have spread rapidly in clinical analysis for the measurement of hormones, drugs and steroids, and then viruses and microorganisms. However, food science and environmental analysis have slowly realized the benefits of this methodology and only within the last 5 years has it been considered as a valuable new quantitative technique and an alternative to the more conventional analytical procedures: GC-MS, HPLC, and microbial growth assays.[4] Immunoassays are not a panacea, but they are assuming increasing importance and value in routine food analysis. The technique is now gaining acceptance by Public Analyst and Quality Controllers, and many investigations are being carried out to offer the easiest, fastest, most accurate, and cheapest methods.

Centrifugal analyzers and air-segmented continuous flow systems are popular instruments in routine use for automated immune-based assays,[5] but both are relatively expensive and of complex design. Other attempts have been made by using a nonsegmented continuous flow system such as flow injection analysis (FIA). Flow injection analysis — a relatively new technique described by Ruzicka and Hansen in 1975 — has proven to be applicable to a wide variety of analytical problems.[6] The FIA technique is based on the controlled and reproducible dispersion of a sample zone when it is introduced into an unsegmented continuously flowing carrier stream, and provides an attractive alternative for the automation of classic methods. The wide success of this method is due to several features, of which the most important are (1) its extremely high flexibility in adapting most chemical and biochemi-

cal reaction procedures; (2) its compatibility with virtually any detection method; and (3) its reliability in low volume, rapid experiments, allowing application in on-line monitoring of chemical processes. Also, the possibility to perform different FIA manifolds, the compatibility of on-line reactors (e.g., immobilized enzymes) and on-line sample treatment (e.g., dialysis), ease of automation, and simplicity of operation offer additional advantages of particular interest for the study of biochemically selective interactions.[7] It is not surprising that the advantages inherent to FIA also have led to a large number of applications in the field of life sciences.[8] The most common biochemical reactions monitored by FIA are enzymatic assays, where the selective reagent is either in solution or immobilized in open tubular or packed reactors.[9] However, the concept of an antibody-supported flow injection system has received relatively little attention. Only recently has the advantage of the specificity inherent to most immune reactions been exploited in FIA procedures.

Flow injection immunoassay (FIIA) techniques provide complementary and/or alternative approaches to reducing the use of costly, sophisticated equipment and test time, maintaining reliability and improving sensitivity. Obvious difficulties in applying FIA techniques to immunoanalysis are (1) the relatively low progression of immunological reactions and (2) quantitative detection. A large number of detection systems have been described in the context of these analytical procedures. At first, optical assays such as photometric, fluorimetric, and luminometric prevailed, but more recently many electrochemical determinations also have been tested to try to solve the above-mentioned difficulties. In this article, emphasis is put on the compilation of FIA procedures involving antibodies. We try to summarize the present state of the art of flow injection immunoanalysis (FIIA).

II. FLOW INJECTION IMMUNOASSAY TECHNOLOGY

A wide variety of methods using FIA have been developed for visualizing the primary antibody-antigen reaction. Following these, FIIA methods are reviewed.

A. Immunoprecipitation

One of the most specific biochemical reactions is the "immunoprecipitation" between a protein (an antigen) and a specific antibody. The basis for the determination is the formation of aggregates when the bivalent antibody molecules combine with the usually multivalent antigen molecules.[10] The reaction is observed directly without the use of any tagged reagents for amplification. Antibodies bridge between antigens to form large insoluble supramolecular structures. These aggregates scatter light appreciably, and the increase in the absorbance (turbidity) is then measured.

In 1972, the immunoprecipitation reaction was used to develop the Laurell's method[11] to determine the concentration of specific proteins by electroimmunoassay. However, the technique has two serious drawbacks: it demands much manpower and it takes 2 to 12 d before the results are available. The current importance of the immunoprecipitation technique for the analysis of protein has been emphasized by the development of an immunoprecipitation analyzer[12] and the subsequent use of laser nephelometry to increase the sensitivity of the method.[13] Mechanization of the analytical process would be advantageous.[14,15] The FIIA immunoturbidimetric technique is rapid (40 samples per hour) and cheap (1 μl antiserum per assay). The merging-zones, stopped-flow procedure is applicable to the determination of most clinically significant serum proteins and some therapeutic drugs.[16]

The suitability of FIA for the study of biochemical specific interactions has been discussed and their possibilities have been illustrated by the analytical performance of a model system.[17] This work describes a stopped-flow system for monitoring the precipitation interaction between yeast mannan (antigen-like molecule) and Concanavalin A (antibody-like molecule) by using a merging-zones approach. Concanavalin A and yeast mannan are introduced simultaneously into separate buffer streams. The system is operated in the stopped-flow mode by using an electronic pump timer and the turbidity is monitored at 420 nm with a spectrophotometer. The relationship between antigen concentration and turbidity has a relative standard deviation (RSD) <3%, between 0.1 and 10 mg l^{-1} under the established conditions. The same biological

model was used in a stopped-flow, merging zones FIA manifold.[18] The simple manifold design can be modified easily to accommodate a wide range of chemical reactions, on-line processes, and detection systems. The use of stopped-flow merging zones minimizes the consumption of sample (30 µl) and reagents, and ensures complete mixing. For the Concanavalin A-yeast mannan reaction, the sample rate is 50 samples h^{-1} and a typical correlation coefficient (r) is 0.9808 for the analytically important antibody excess region of the calibration graph (yeast mannan concentrations in the rage 0.0 to 0.4 mg ml^{-1}). The use of a single-channel manifold offers enhanced sensitivity and clearly distinguishes between antibody excess and antigen excess. This technique would be applicable to routine immunoprecipitation analysis for the determination of human immunoglobulins G(IgG) in serum, and work is currently being carried out in this area.

Several instrumental methods have been developed for serum IgG determination, based on the light-scattering ability of the antibody-antigen complex. These include a rate nephelometer method,[10] a segmented continuous flow technique using an automated immunoprecipitation analyzer,[12] and a centrifugal analyzer procedure.[19] The flow injection (FI) technique has been shown to provide a cheap, rapid, and automated analytical facility that is also extremely flexible in its application. An automated merging-zones FI procedure for the determination of human serum IgG via its interactions with goat anti-human IgG, and based on rate turbidimetry, has been reported.[20] Antigen samples are automatically introduced into the injection valve via an autosampler, and a separate computer-controlled peristaltic pump is used to introduce the antibody into the injection valve. The linear range is 0 to 3556 mg $d\ell^{-1}$ with a 2 to 4% RSD and a sample frequency of 40 samples per hour. The feasibility of using FI to monitor immunoprecipitation interaction was demonstrated and the results compare favorably with radial immunodiffusion. A fully automated version of the above-described FI manifold was developed by Worsfold et al.[21] A sampling rate of 40 samples per hour and a precision of 2.0 to 6.8% RSD are obtained for a range of 889 to 1778 mg $d\ell^{-1}$ human serum standards. Serum samples and human reference serum were analyzed and their IgG concentrations interpolated from a second-order fit of the calibration data. The results show that the stopped-flow, merging-zones FI technique is suitable for the determination of human serum IgG using rate turbidimetric detection, and no sample pretreatment, other than dilution, is needed. By changing the serum and antiserum dilutions, the technique can be extended to the analysis of several other plasma proteins and certain therapeutic drugs.

Based on these experiences,[16,20,22] several attempts have been made to determine specific proteins in plasma using an FIAstar analyzer (Tecator) with two-channel injector.[23] The following seven specific proteins were investigated: urine albumin (u-albumin), plasma transferrin (*p*-transferrin), *p*-haptoglobin, *p*-IgG, *p*-IgA, *p*-IgM, and *p*-orosomucoid. The prediluted plasma samples and antibodies are allowed to react for 33 s before the change in turbidity is measured. The throughput is nearly 38 determinations per hour. Results are available within an hour, compared with 2 to 12 d with the electroimmunoassay method. The FIA method setup described can be used for u-albumin, *p*-transferrin, and *p*-haptoglobin. For *p*-IgG, *p*-IgA, and *p*-IgM, the 33-s reaction time is insufficient because their relative molecular masses are higher. In this case, FIAstar is not suitable because of the much longer reaction times (5 to 10 min). For *p*-orosomucoid, the FI principle can be used if the FIA analyzer measures the reaction kinetically.

Recently, a system for real-time monitoring of specific proteins in fermentation processes was introduced.[24] The FI principle is used to fully automate the assay, including calibration and washing steps. The analyzer is used to measure monoclonal antibodies (MAb) produced in fermentation of mouse-mouse hybridoma cells as well as to quantify thermosulfurogenes. The turbidity caused by aggregates formed between the proteins to be detected and their antibodies is measured photometrically at 340 nm. The assay is optimized to cover concentrations ranges of 1 to 1000 mg ml^{-1} and a RSD of less than 2% is obtained. Correlation coefficients of 0.94 and 0.99 are found between the on-line assay and the reference assay (conventional ELISA).

An automated FI for on-line analysis of proteins in real fermentation broths was developed,[25] combining the principles of stopped-flow, merging-

zones FIA with antigen-antibody reactions. This system was used to monitor MAb production in high cell density perfusion cultures of hybridoma cells. Different sampling devices were tested to obtain a cell-free sample stream for on-line product analysis of high molecular weight medium components. In fermentation fluids, a good correlation ($r = 0.996$) between the FIA method and an ELISA test was demonstrated. In a high-density perfusion cultivation process, MAb formation was successfully monitored on-line over a period of 400 h using a reliable sampling system. The experimental results indicate that the FIA immunoassay system allows for on-line monitoring of product formation in continuous, long-term cultivation processes, thus facilitating routine operation and safety supervision. The application of FIA offers new possibilities for the development of production strategies.

Finally, an on-line assay for a thermostable pullulanase and antithrombin III (AT III) was described.[26] Assay automation was achieved by using a stopped-flow, merging-zone FIA manifold. Because the reaction was coupled with a FIA system, it was possible to reduce the assay cycle time to 2.5 min. A method for simulating cultivation conditions was developed for assay optimization. Using this method, a detection limit of 1 mg l^{-1} together with a standard deviation of 1.5 was found. Correlation coefficients of 0.988 (AT III) and 0.976 (pullulanase) were obtained.

B. Fluoroimmunoassays

1. Homogeneous

a. Fluorimetric Detection

The first example of FIIA proposed a homogeneous fluorescence immunoassay for serum albumin based on the principle of nonradioactive energy transfer.[27] Fluorescent energy transfer immunoassay is one of a number of types of competitive binding assay introduced in order to avoid the use of isotopic labels. The assay is based on the transfer of excitation energy from the labeled antigen or hapten (X*) to the labeled antibody, which only occurs when they are in specific combination. This results in quenching of the fluorescence of the label bound to X and enhancement of the fluorescence of the label bound to the antibody. These effects are reversed when an unlabeled antigen is present. Fluorescein and Rhodamine were used as the donor and acceptor labels, respectively, and fluorescence intensities were measured at 541 nm, using 470 nm as the excitation wavelength. Fluorescein-labeled albumin, on binding to Rhodamine-labeled antialbumin antibodies, suffered a reduction in the albumin-antibody complex. A reagent volume of 36 µl and an incubation time of 6 min at 37°C were adopted. Using merging-zone and stopped-flow principles, the detection limit is about 10^{-7} *M*, and the sampling frequency is 10 h^{-1}. The method is precise, with a RSD of 2.5%.

Measurements of drug-protein binding interactions are important in pharmacology, and there is an increasing demand for rapid, simple, and sensitive methods for the study of systems in which two or more drugs compete for the binding sites on a single protein such as serum albumin. 8-Anilino-naphthalene-1-sulfonic acid (ANS) and warfarin have been used as molecular probes.[28] The authors used the same merging-zones system mentioned above,[27] although it was found that a delay coil was unnecessary in many circumstances. The very large enhancement of ANS fluorescence that occurs when this molecule binds to serum albumin had already been established. If, however, ANS is added to albumin together with a drug molecule that competes for the ANS binding sites, the fluorescence enhancement effect is reduced. The same phenomenon occurs on a less dramatic scale when warfarin is used as the probe.

In other work,[29] a fluorescent product analogue of the drug propanolol (DAPN), whose dimethylaminonaphthalene sulfonyl ("dansyl") group acted as a fluorescence probe, was assayed. The fluorescence intensity of this molecule is enhanced about eightfold on binding to orosomucoid. The fluorescence enhancement effect is reversed by the presence of basic drugs that displace the DAPN from the protein binding sites. In the case of the DAPN-orosomucoid interaction, it was shown that a static fluorescence analysis required several hours of work and used about 30 ml of solutions of each material. A conventional single-channel FI system used about 0.3 ml solution and took about 1 h, whereas a gradient FIA took about 300 s and used 30 µl of solution.

b. Other Detection Systems

Many authors have undertaken the logical step of combining FIA with chemiluminescence (CL) detectors. FI can provide the extreme reproducibility of sample and reagent mixing that is so essential for precise CL studies. FIIA with CL detection has been applied to several systems. A reaction involving *bis*(2,4,6-trichlorophenyl)oxalate (TCPO) is the most commonly used. This reaction is of great potential value in biochemical systems where fluorescent labels are readily bound to analytes (e.g., fluoroimmunoassay). Such labels can be excited and detected with higher sensitivity and simpler equipment than in conventional fluorimetry, and the substantial background signal caused by scattering of the external light source in a fluorimeter does not arise in chemiluminescence measurements. Other oxalate compounds also have been used (e.g., peroxyoxalate). However, a disadvantage of these systems is that the oxalate esters that are most useful as sources of CL are not soluble in water, so that mixed solvent systems will normally be necessary in biochemical studies where a mainly aqueous environment must be maintained.

The combination of FIIA with a CL detector to study fluorescein and fluorescamine-labeled species at concentrations as low as 10^{-1} *M* (ca. 0.5 pg in a 100-µl sample) was carried out.[30] The effects of antibodies on the luminescence signals from labeled antigens are discussed. Preliminary experiments with a fluorimeter as the detector showed the high sensitivity of the technique. When fluorescein was mixed with 5 m*M* TCPO and injected into a flowing stream of peroxide, 10^{-14} mol of fluorescein could be detected. Fluorescein-labeled albumin showed much less emission. Fluorescamine-labeled thyroxine and triiodothyronine showed detection limits at least as good as those obtained with the best fluorescence spectrometers. Furthermore, two important effects that would limit the applicability of the assay in biological samples have been revealed. First, it was apparent that the premixing of sample and TCPO could lead to a signal decrease, which may be explained by the hydrolysis of TCPO in aqueous media. To eliminate this effect, more sophisticated merging-zones systems for FIA are required. It was also observed that the addition of TCPO in acetone to the phosphate buffer produced a turbid solution with a high absorbance. Only a 1:1 acetone to water mixture, which would be unacceptable in immunoassay, would eliminate this effect.

This work demonstrated the potentially great sensitivity of combining peroxyiodate reaction with FI systems for the determination of these fluorescent derivates. The CL signal is, however, subject to nonspecific quenching. Preliminary studies indicated that such effects, and the problems caused by the poor solubility of TCPO in aqueous systems, can be minimized by a careful choice of buffer systems. Homogeneous immunoassays utilizing fluorescence enhancement effects can then be developed successfully.

2. Heterogeneous

A reusable packed bed reactor with agarose-bound protein A, which binds to antibody molecules at sites separate from those at which antigens combine and thus allows the antibody components of an immunoassay mixture to be removed from unreacted antigen and sample matrix species, has been proposed.[29] The system is formed by a reactor containing swollen beads of protein A-agarose and a fluorescence detector with flow cell. Immunoassay incubates were injected into this FIA system in a Tris-HCl buffer and a manually operated switching valve was used to introduce potassium thiocyanate and thus remove bound antibody from the reactor after each sample injection.

Trial injections of fluorescein-labeled goat antibodies yielded two fluorescent peaks per sample. This result was expected because goat IgG2, but not goat IgG1, binds to protein A. However, twin peaks were also obtained when labeled rabbit antibodies were injected. Because all these antibodies should, theoretically, bind to protein A, this result indicated that either a short residence time in the reactor, or the blocking of protein A binding sites on the antibody by the fluorescent label groups, prevented complete binding. When the fluorescence intensity of the fraction not bound to the protein A reactor was determined, the expected increase of fluorescence with sample albumin concentration was found, and the assay could readily be used to determine submicromolar albumin concentrations. The protein A reactor could be used in this alternating fashion for hundreds of samples over several days.

Recently, helical-shaped magnetotactic bacteria have been cultured and isolated from an aerobic Fe-containing medium.[31] These cells have oxidase activity and have been used to determine mouse IgG. When the cells were disrupted using ultrasonication, 2.6 mg bacterial magnetites were obtained from 1-ℓ culture of magnetotactic bacteria. The detection of mouse IgG was carried out using fluorescein isothiocyanate-conjugated anti-mouse IgG immobilized on bacterial magnetites and a FI system with a fluorescence spectrophotometer. Relative fluorescence intensity correlated linearly with the concentration of mouse IgG in the range 0.5 to 100 ng ml^{-1} and the measurements were established within 2 min using this system.

C. Enzyme Immunoassay

Enzyme immunoassay (EIA) uses enzyme markers to amplify and visualize the primary antigen-antibody binding reaction and is likely the most rapidly growing and the most widely used immunoassay technology today. The use of enzyme tags in immunoassays was introduced early.[1,2] These labels are easy to handle, inexpensive, and stable, and are used to convert a colorless substrate into a colored product providing sensitivity and simplicity to interpret endpoints. The suitability of an antibody preparation in an immunoassay is governed by the affinity with which it binds the corresponding antigen or hapten, and this affinity must be evaluated separately before an immunoassay protocol is established.

Two general approaches are available to follow these reactions. The most widely used one is the heterogeneous assay, in which the molecular complex formed is physically separated from the uncombined molecules before determination. The activity of the enzyme label is not affected by the antigen-antibody reaction. This method has two advantages: it is universally applicable and the separation step removes potentially interfering matrix components before the final measurement of the complex. However, the separation step may be complicated in practice, especially if automation is desired. The alternative, homogeneous, assays utilize a probe molecule whose properties change when a ligand binds to the corresponding receptor. The extent of this change can be used as a direct measure of the complex formation and no separation step is needed. However, an easily measurable change of property cannot always be obtained, and the nonreacting matrix components are still present at the measurement stage, which may cause interferences.

1. Homogeneous

Homogeneous EIA — EMIT — has been used extensively in clinical diagnostics for detecting low molecular weight compounds such as drugs and hormones. This method has the advantage of giving a positive dose-response curve.

a. Fluorimetric Detection

A homogeneous immunoassay for serum IgG using peroxidase as the enzyme label and H_2O_2 peroxidase-catalyzed oxidation of *leuco*-diacetyldichlorofluorescein as the indicator reaction has been proposed.[32] Serum IgG concentrations from 1.4 to 25 mg ml^{-1} could be determined with a sampling frequency of 60 samples per hour. The binding of the labeled antibody to the antigen, IgG in this case, partially inhibits the enzyme activity, resulting in a decrease in the fluorescence intensity produced by the indicator reaction. A dual-channel FIA system automatically performs the appropriate sequence of reactions before the detection by a laser filter fluorimeter with a sheath flow cell.

Allain et al.[33] reported an automated determination of theophylline and valproic acid by use of Ames' fluoroimmunoassays performed with standard high-pressure liquid chromatography (HPLC) instruments, used without a column in a FIA mode. The results correlated well with those of conventional liquid and gas chromatography ($r > 0.96$). The between-run coefficient of variation (CV) is about 5%. In comparison with the manual method, the volume of reagents was decreased by eightfold.

b. Other Detection Systems

Other detection procedures for homogeneous enzyme immunoassays include colorimetry, electrochemistry, spectrophotometry, and chemiluminescence.

In the homogeneous immunoassay with colorimetric detection developed by Kelly,[34] IgG is determined by its reaction with the antibody, goat anti-IgG. Antibody-conjugated horseradish peroxidase (HRP) is partially inhibited by antigen-antibody binding from catalyzing the reaction of hydrogen peroxide with activated dye, and thus can serve as a means of detection in the FIA system.

A flow-injection amperometric system for immunoenzymatic analysis was reported by Ivnitskii et al.[35] The system comprises a peristaltic pump for the electrolyte, a sample dosing valve, and an amperometric detector. The latter includes a tubular indicator electrode and a Ag-AgCl reference electrode, coupled to an amplifier and a potentiometer. The system has been used for the determination of α-amylase at the microgram per milliliter level.

It is known that the FIA technique is not ideally suited to clinical use.[36] The limitations of conventional FIA were overcome by developing an alternative method of sample introduction suitable for clinical chemistry applications.[37] In this technique, which is referred to as controlled-dispersion flow analysis, injection valves are not used, and the sample is aspirated directly into the nonsegmented stream. Thus, none of the sample is wasted. To illustrate the versatility of controlled-dispersion flow analysis, a description of its applications to three very different but established clinical analyses was done.[38] The determinations of albumin, triglycerides, and theophylline were chosen to emphasize different aspects of this new approach. The authors adapted the EMIT kit for use as an analyzer. An antibody to theophylline is first added to serum and binds to any theophylline present. Enzyme-labeled drug is then added and will bind to any sites on the antibody not already filled by theophylline. Only the free, enzyme-labeled drug can react with the enzyme substrate, resulting in the oxidation of the coenzyme NADH and hence a change in absorbance at 340 nm. Because the antibody and the labeled theophylline cannot be included in the same reagent, a two-reagent merging-zone system was employed. The manifold tubing was arranged so that the reagent containing antibody, enzyme substrate, and coenzyme was added to the sample plug before the second reagent containing labeled drug. The sample volume was 9 μl and the linear range obtained was 2.5 to 40 mg^{-1}. This work demonstrates the great flexibility of controlled dispersion flow. Determination requiring the addition of more than one reagent also may be used in the merging-zone mode using controlled-dispersion flow analysis.

Finally, based on the enhanced chemiluminescent reaction, a peroxidase FI assay was developed and tested successfully by Vlasenko et al.[39] for human IgG and thyroxin. The FIIA proposed has a detection limit of 10^{-9} *M* for IgG and 10^{-11} *M* for thyroxin, the overall time of the assay being 5 to 15 min.

2. Heterogeneous Competitive ELISA

The ELISA technique can be performed in two formats, either a competitive equilibrium scheme or a "sandwich" scheme. Competitive ELISAs can be divided into direct and indirect. Direct ELISA uses primary antibodies immobilized onto a solid support and an enzyme-labeled antigen conjugate. In the indirect competitive ELISA, the antigen is immobilized onto the solid phase. Both assays are widely used for analysis of small molecules such as antibiotics, pesticides, toxins, and hormones. Only direct competitive ELISAs using flow injection methods have been reported.

a. Electrochemical Detection

The application of electrochemical (EC) detection in EIA is under active investigation because of combined low detection limits and good selectivity. Both potentiometric and amperometric methods have been developed to be used with homogeneous and heterogeneous assays.[40] Coupling of FIIA to electrochemical detection (FIIA-EC) is also being investigated, which will permit the development of more sensitive assays.[41]

The most popular electrochemical cell design used in FI techniques is the thin-layer type, which has excellent flow characteristics and a high ratio of electrode surface area to cell volume for high response with low dead volume. EC immunoassays are based on labeling the antigen with an enzyme that catalyzes the production of an electrochemically detectable product. The rate at which the product is formed is related to the concentration of the analyte in the sample.

Several ELISAs with EC detection have been reported,[42–46] for example, a FIIA-EC for the determination of digoxin in human plasma.[43] Digoxin (hapten) in the sample or standards and digoxin labeled with alkaline phosphatase (labeled hapten) compete for solid-phase antibody coated on the walls of reagent tubes. Unbound digoxin and labeled digoxin are then rinsed from the tubes. Labeled digoxin bound to the solid-phase antibody is determined by incubation with the enzyme substrate solution (phenylphosphate). The phenol produced by the enzyme reaction is quantified by oxidative hydrodynamic amperometry in a thin-layer cell using either FIA-EC or liquid chromatography-electrochemistry (LC-EC). FIA-EC involves the direct injection of the sample into a thin-layer electrochemical cell, while in the LC-EC approach the phenol is retarded by a C-18 column. A good correlation between this method and RIA was obtained for determinations in patient's samples. The FIA-EC and LC-EC methods offer different advantages: LC-EC has a 100-fold better detection limit than FIA-EC (detection limits 5.10^{-9} and 5.10^{-7} *M*, respectively). However, this advantage is gained at the expense of a longer analysis time (2.5 min for LC-EC compared with 25 s for FIA-EC). FIA-EC could not be used in the shortened assay format because the amount of phenol produced for all digoxin standards under these conditions is too close to the detection limit of the technique.

Some immunosorbent microreactors aimed at achieving a higher effective concentration and faster reaction rates have been described. One used Fab' fragments of an antibody attached covalently to a polymeric support (Trisacryl GF-2000), and coupled to FI with a thin-layer EC detector.[44] A FIIA for human IgG was carried out with ±2.3% precision and sensitivity in the subpicomole range. The total time taken for the assay was 12 min and the technique was compatible with total automation.

An ELISA using flow-injection amperometric detection of *p*-aminophenol (pAP) has been investigated.[45] The authors assessed the feasibility of using FIA-EC in the EZ-BEAD (Immunotech Corp., Boston, MA 02134) procedure for a theophylline assay by using *p*-aminophenyl phosphate (pAPP) as enzyme substrate to generate pAP, which is detected by its oxidation current at a glass electrode in the FIA-EC system. The current generated during the EC detection allowed a rapid (35 min) and simple determination of theophylline with a detection limit of 80 μg l^{-1}

The pH of the system was discussed[46] because the pAP oxidation is faster at the higher pH necessary for the optimum activity of alkaline phosphatase. Serum samples from patients under theophylline therapy were assayed by FIA-EC and by the Abbott T Dx kit. A good correlation was obtained ($r = 0.98$) and the detection limit of both procedures was about 1.2 mg l^{-1}. Furthermore, the better sensitivity of the FIA-EC approach results in saving about 50% in reagent cost and in the ability to work with a 0.25-μl sample volume in faster assays.

b. Fluorimetric Detection

The production of MAb in the course of mouse hybridoma cell fermentations is usually monitored by classic competitive or sandwich ELISA. In recent years, however, some examples of the application of FI techniques for the on-line monitoring of those bioreactors have been described.[47–49] Mouse IgG or anti-mouse IgG are attached on a solid phase (Immunodyne membrane or magnetic particles) and an antibody-peroxidase conjugate also is used, the product of the enzymatic indicator reaction being measured fluorimetrically. MAb can be determined in the concentration range of interest in hybridoma cell fermentation.

The proposed FIIA manifolds follow the ELISA principles, but instead of using a microtiter plate, the reagents and washing buffers are pumped consecutively through the reactor containing immobilized antigen. The competitive assay is performed in two ways:

1. On *Immunodyne membrane:* a membrane piece containing 15 μg of antigen protein in a 1 cm^2 effective area is applied per assay. Antigen and conjugated antibody are injected via a double injection valve into the carrier stream, mixed, and incubated for 1 min in the incubation coil before passing through the reactor containing immobilized antigen. Then, the substrate of the enzyme reaction [3-(*p*-hydroxyphenyl)propionic acid] and hydrogen peroxide are injected via a second valve and allowed to react for 2 min

with the bound peroxidase. Finally, the fluorescence of the product is detected. The detection limit is 0.1 to 0.5 μg ml^{-1} antigen and the linear range 1 to 100 μg ml^{-1}. The cycle time is 15 min per assay and the RSD in absence of free antigen is 5%. The remaining fluorescence in the presence of excess antigen is 40% of the maximum, due to nonspecific binding of the conjugate to the membrane and FIIA tubes. The amount of immobilized antigen should be high enough to allow a specific binding of conjugate over nonspecific binding.

2. On *BioMag 4100:* the procedure is the same as in the competitive membrane FIIA except that the amount of immobilized antigen in a 20-μl magnetic reactor is 9 μg. The dynamic range and the background are the same as those obtained with the membrane system. Presumably, differences for the physical and chemical properties of the solid phases and reactors compensate for differences in the bound protein contact. The same assay has been extended to the determination of different subclasses of mouse IgG compared with a polyclonal mouse IgG standard.[50] A sampling frequency of 1 sample per hour affords FIA cycle times of 30 min or shorter, corresponding to an incubation of 6 min or less for both the analyte and the conjugate binding steps.

Recently, the appearance of pesticide residues in ground water has caused considerable problems in the control of drinking water quality. Regulations governing pesticide concentration in drinking water are strictly enforced, with a maximum allowable pesticide concentration of 0.1 μg l^{-1} per pesticide. The great number of pesticides to be evaluated at critical concentrations makes it difficult to find an ideal method. Usual techniques for controlling pesticides are HPLC and GC/MS, but these methods are expensive and time consuming. Therefore, increasing efforts are being devoted to the development of fast, automated, and inexpensive tests for routine analysis in water supply stations and control laboratories.

The first results illustrating the possibilities of using FIIA in pesticide determination were described for herbicides of the triazine group.[51–54] Atrazine and aminohexylatrazine were assayed, the results showing a good correlation with the competitive ELISA. Therefore, FIIA is proposed as a potential alternative to chromatographic methods. Little variation in this FIIA system to determine triazine pesticides has been introduced.[55–57] The reagents are pumped one after another in a "stop and go" sequence in a cross-flow over the membrane with antibodies. The peroxidase substrates are hydroxyphenyl propionic acid and H_2O_2. The enzyme-generated product is then measured downstream in the flow-through cuvette of a fluorescence detector, the result being inversely proportional to the pesticide concentration. For triazine, the detection limit is 0.02 μg l^{-1} and the linear range is 0.02 to 0.3 μg l^{-1}. For simazine, the detection limit is about 0.08 μg l^{-1} and measurements should be carried out between 0.08 and 1 μg l^{-1}. It is also possible to measure other herbicides of the triazine group (such as propazine) with appropriate antibodies. Immunoassays for different pesticides have been under development for about 10 years. However, until now it has not been possible to obtain the pesticide-specific antibodies or the haptens that are needed to prepare enzyme conjugates to be used in this competitive test. This means, in fact, that the needed reagents are not available in "unlimited" amounts. On this side of the development, it is necessary to get the chemical industry involved.

c. Chemiluminescent Detection

A type of FIIA that uses acridinium-labeled antibodies for detection has been developed.[58] The entire assay, including detection, takes place in a transparent 20-μl immunoreactor consisting of antibodies immobilized onto a polymeric bed support. Both a competitive and a two-site heterogeneous immunoassay can be conducted. The two-site assay is accomplished by the respective injection of the sample, acridinium-labeled antibody, and the chemiluminescence initiation reagent. The emitted light is collected directly from the transparent immunoreactor, which is then regenerated to prepare the next sample injection. In the competition assay format, the sample containing the antigen being measured is mixed with acridinium-labeled antigen before injection into the immunoreactor. Competition for antibody sites in the immunoreactor occurs and the signal is inversely proportional to the amount of analyte present. A two-site immunoassay for mouse

IgG requires 12 min per sample and has a detection limit of 200 amol. The intra-assay CV is less than 5%. A competition immunoassay for digoxin is under development and is also discussed.

A nonequilibrium FIIA for thyroxine involving separation of the immunocomplex of HRP-labeled antibodies and antigen from the free labeled antibodies has been described.[59] In this procedure, the antigen to be analyzed is sampled into the buffer stream and then interacts with a large excess of HRP-labeled antibodies to ensure a fast and almost complete binding of the antigen. The unbound labeled antibodies are removed by passage through an affinity column containing the antigen immobilized in large excess with respect to the antibodies. The column shows no loss of activity after 100 experiments. An enhanced CL reaction is used for the detection of HRP involved in immunocomplexes. The method allows the determination of thyroxine concentrations as low as 10^{-11} *M* within 5 min and the precision is 10%. The proposed FIIA method with enhanced CL detection is fast, reproducible, convenient for routine flow immunotesting, and characterized by a high sensitivity with respect to antigens.

d. Spectrophotometric Detection

The analysis of transferrin in human plasma using an immobilized preparation of rabbit anti-human transferrin antibodies and HRP-labeled transferrin was studied.[60] A competitive assay is set up wherein enzyme-labeled antigen competes with native antigen for a limited number of binding sites on the antibody column. Two particular aspects are stressed: the appropriate amount of enzyme-labeled antigen and the time required for the splitting of bound antigen from the antibody column before reconditioning the column. Transferrin can be quantified in the concentration range of 25 to 750 $\mu g\ ml^{-1}$. Two advantages of this FIIA are quickness (7-min assay cycle time) and no recalibration needed (the calibration curve remains valid during the whole lifetime of the antibody column).

A fully automated flow system has been developed to control bioreactors and downstream processing monitoring.[61] The antibodies are immobilized on a solid support and placed in a small column in the flow system. After one assay cycle, the system is rinsed and the column is reused for the next assay. The total time for an assay cycle is 6 to 10 min, depending on the conditions. The whole procedure is controlled by a personal computer, with the sampling, addition of labeled antigen, washing, monitoring, evaluation, dissociation, and reconditioning being computer controlled. To evaluate the system, a model interaction was studied. Protein A-Sepharose was packed in the column and rabbit IgG was determined. HRP-labeled rabbit anti-human IgG was used as the competing marker. The absorbance was monitored at 280 nm and the assay cycle was repeated 71 times. A decay in the measures indicates a decrease in the capacity of the column. It is possible to run reliable assays over long time periods provided that proper compensation for denaturation is accounted for. The program is designed to compensate for denaturation of antibodies on the column. This flow-ELISA was suitable for on-line monitoring of biological macromolecules.

e. Other Detection Systems

The detection of mouse IgG with an enzyme thermistor was illustrated for on-line monitoring of real fermentation processes.[62] The concentration of mouse IgG was measured calorimetrically in a heterogeneous competitive assay. Immobilized antibodies were packed into the enzyme column. Samples containing enzyme-labeled mouse IgG in different concentrations were aggregated prior to the addition of substrate. The more IgG bound, the more enzyme activity that could be detected. This flow-through system with a sample processing time of about 6 min offers interesting aspects for protein monitoring during fermentation processes.

3. Competitive ELISA-Second Antibody

New FIIA systems making use of immobilized second antibodies to separate bound and free enzyme-labeled analyte in a flowing sample solution have also been described. The sample containing the analyte is first equilibrated with given amounts of enzyme-analyte conjugate and first antibody. Because this antibody is added as a soluble reagent, its effective concentration can be controlled and its

natural affinity and homogeneity are preserved. After a brief nonequilibrium incubation period, the reaction mixture is injected through the immunosorbent reactor containing an excess of second antibody (an antibody that selectively binds the first antibody). A fixed fraction of the total first antibodies binds to the column with associated analyte or enzyme-labeled analyte. All unbound species are washed away by the carrier stream buffer. Enzyme activity bound to the reactor can be detected by diverting a flowing solution of substrate through the reactor and monitoring the product downstream with an appropriate flow-through electrochemical or photometric detector. After detecting bound activity, the reactor is regenerated by diverting a low pH buffer through the column for a brief period. The flow of various solutions through the system is controlled by several rotary injection valves.

A FI assay was described[63] for the determination of insulin or 17-α-hydroxiprogesterone using glucose oxidase (GOD) as the label. The enzyme activity was measured by the chemiluminescence produced by luminol and hydrogen peroxide, catalyzed by potassium hexacyanoferrate (III) after incubation with glucose. Free and bound fractions present after the immune reaction were separated by an immobilized second antibody. Both analytes can be determined in the range 10^{-17} to 10^{-15} mol per assay. The sensitivity of this method is 10 times higher than that of the fluorescence immunoassay for insulin. Owing to the great sensitivity of the CL methods, only a few microliters of sample are needed for each determination. Moreover, when the antibody solid-phase technique is used for separation of bound and free fractions, interfering substances in biological samples can be eliminated completely by washing.

Another fast FIIA system using an immobilized secondary antibody reactor was introduced by Lee and Meyerhoff.[64] Enzyme-labeled antigen and free antigen are mixed with primary antibody and then introduced via flow injection into the secondary antibody reactor. The reactor-bound enzyme activity, measured by flowing an appropriate substrate solution through the reactor, is inversely proportional to the concentration of free analyte in the sample. To illustrate the application of the system, theophylline and insulin were chosen as model haptен and macromolecule analyte, respectively. In the case of theophylline, adenosine deaminase was used as the labeling enzyme and its activity was detected downstream with an ammonium ion selective electrode. For insulin, HRP was employed as the label with photometric detection. Sheep anti-theophylline and guinea pig anti-insulin were used as first antibodies, while the reactor contained immobilized anti-sheep whole serum and anti-guinea pig whole serum, respectively. The working range for theophylline is 0.025 to 0.25 μ*M* and 1 to 250 μg ml^{-1} for insulin. Detection limits in these FIIAs were controlled by the amount of soluble first antibody required to bind an amount of enzyme conjugate that yielded a significant detector signal upon flowing substrate through the reactor.

Using more sensitive detection methods, fully automated reagent dispensing, and valve control, this heterogeneous enzyme-linked FIIA system could become an attractive approach for performing heterogeneous FIIAs on a routine basis.

4. Sandwich ELISA

This widely used reagent configuration is applicable to the measurement of bivalent and polyvalent antigens and is often referred to as a sandwich assay because the analyte is sandwiched between the solid-phase antibody and the enzyme-labeled antibody. The primary antibody is immobilized on a solid support and is exposed either to known standards or test samples. After rinsing away the unbound material, the immobilized antibody-antigen complex is exposed to an excess of the enzyme-labeled antibody that will attach to the remaining determinant sites on the antigen. After a final rinse step, the enzyme substrate is added. The signal produced is proportional to the amount of antigen present in the sample.

The sandwich assay is highly sensitive and precise compared with other heterogeneous EIAs, but it requires long incubation periods. When sandwich immunoassay is combined with FIA, the procedure consumes less reagent because immobilized analytical reagents often may be reusable. FIA-based sandwich assay offers greater speed (less than 10 min) and precision (better than 5%) than conventional ELISA assays using microtiter plates or plas-

tic tubes. This improvement is due to the much better reproducibility associated with reactions of solid-phase components and soluble species.

a. Electrochemical Detection

The combination of analytical techniques such as LC-EC or FIA-EC with enzyme immunoassay is frequently able to fulfill the needs of the clinical laboratory and research community. EC EIA methodology incorporates the specificity and sensitivity of the antibody-antigen reaction, the fast turnover rate of substrate to an electroactive product under enzyme catalysis, and the capability of detecting a small sample volume by LC-EC and FIA-EC.

One of the first models for EC sandwich immunossay was performed by including a chromatographic step combined with the immunoassay in a continuous flow scheme.[65] In this model, the antibody-like reactant is immobilized on a short chromatography column (immunoreactor). The antigen sample is injected into the reactor where binding occurs, and the enzyme-labeled second antibody is then injected. Finally, the substrate solution is injected and the product detected electrochemically by means of a thin-layer amperometric detector. The amount of electroactive product formed is proportional to the amount of antigen bound to the column. After analysis, an acidic buffer is passed through the immunoreactor to displace antigen and second antibody complex in preparation for the next sample. This technique provides controlled conditioning of the immunoreactor (washing), which is essential for good experimental precision. The technique has been demonstrated for the detection of as little as 1 fmol of IgG using a GOD-goat anti-IgG conjugate as second antibody. The enzyme product, H_2O_2, is detected by oxidation at a platinum electrode. For the calibration curve generated in the range of 3 to 205 fmol, a r value of 0.9997 was obtained. Both inter-assay and intra-assay precision are better than ±3%. In this work, the immunosorbent reactor has been shown to be stable up to 3 months of repeated use (at least 500 assays).

Alkaline phosphatase is a suitable enzyme for EC assays because it catalyzes the conversion of electroinactive phenyl phosphate to electroactive phenol. The product is then quantified by LC with EC detection in a thin-layer flow cell with a carbon paste electrode at 0.895 V vs. Ag/AgCl.[66] The current produced by the oxidation of phenol is directly proportional to the antigen (rabbit IgG) concentration. The problem associated with these types of solid-phase immunoassays is that the adsorption of the primary antibody is desired, while that of other assay proteins is not. The detection limits are generally defined by the ability to control this nonspecific adsorption. The detection limit of a previous EC assay for rabbit IgG was 100 pg ml^{-1} and was limited by a large background current observed in the absence of antigen. In the present study, each step of the assay was examined in order to determine the sources of this background current; it was found that the major contribution was the low, nonspecific adsorption of the enzyme conjugate. Using combinations of Tween 20 and bovine serum albumin as blocking agents, the level of nonspecific adsorption was reduced by 96%. By controlling the adsorption of the conjugate in this way, the detection limit was lowered from the original 100 to 7.5 pg ml^{-1}.

The FIA-EC was also applied[67] to a sandwich-type enzyme immunoassay for mouse IgG, with alkaline phosphatase as the enzyme label. The enzyme substrate, pAPP, and its enzymatic reaction product, pAP, have been studied by cyclic and hydrodynamic voltammetry. The method has shown a wide linear range (5.10^{-8} to 1.10^{-5} *M* pAP or 0 to 6 pg ml^{-1} IgG) and an excellent detection limit (0.81 pg ml^{-1}). The rate sample was 72 h^{-1}. The advantages of detecting pAP are the low oxidation potential and the lack of fouling of the electrode surface, resulting in low background noise and excellent precision.

The same substrate system was used in the determination of α-fetoprotein (AFP) in human serum, pAP being detected amperometrically.[68] For this analyte, a much more sensitive assay than the one currently available is required. An enzyme immunoassay with flow injection amperometric detection for low concentrations of AFP was developed, with a detection limit of 0.163 μg l^{-1} and a linear range of 0.316 to 100 μg l^{-1}. The system consists of an HPLC pump, an amperometric detector, and amperometric flow cell. The amperometric thin-layer cell has a dual glassy carbon working electrode and an Ag/AgCl reference electrode.

Lee and Meyerhoff[69] developed a flow injection sandwich immunoassay system that employs adenosine deaminase as the tracer, together with simple flow-through potentiometric detection of the product of the enzymatic reaction (ammonium ion). The method is based on the sequential sandwich immunoassay technique. After sequential injections of analyte and enzyme-antibody conjugate, unbound species are washed away from the reactor by the flow-injection carrier buffer. After a suitable washing period, a continuously flowing stream of substrate is diverted through the reactor. The amount of bound activity, as measured via a downstream detector, is directly proportional to the concentration of analyte present in the sample. After detecting bound enzyme activity, the immunoreactor can be regenerated by washing with a low pH buffer. In this assay, both human IgG and α-acid glycoprotein were chosen as the model protein analytes. Ammonium ions generated from the enzymatic reaction are detected with a simple and sensitive tubular ammonium ion selective electrode. The advantage of the proposed system is an improvement in assay speed. This is achieved by adapting nonequilibrium flow rates to deliver the sample and reagent solutions. A cycle of the protein assay takes less than 12 min. The proposed method is shown to be selective, reproducible, and able to accurately determine the model protein at submicrogram per milliliter (5 to 400 ng ml^{-1} IgG) concentrations.

Two approaches to FIA-coupled immunosensors were demonstrated[70] for detection of α-interferon (IFN). Specific MAb for human IFN were covalently coupled to an activated nylon membrane and on a Sepharose bead. The membrane covered with capture antibody and saturated with inert protein was placed on a hydrogen peroxide sensor in a flow-through cell. IFN-containing samples injected into the cell formed an antibody-antigen complex that reacted with a second anti-IFN antibody labeled with GOD. After 15 min, the injected glucose reacted with GOD, yielding hydrogen peroxide. The sensor was sensitive in the range from 5 to 50 pmol IFN ml^{-1} (100 ng to 1 μg ml^{-1}). A broader measuring range from 10 to 150 pmol ml^{-1} (100 ng to 3 μg ml^{-1}) was obtained with a 50-μl reactor filled with anti-IFN coupled to Sepharose. As a consequence of the higher antibody loading, the immunoreactor offers the advantage of shorter assay time (30 min) and improved reusability (50 assays without losing binding ability compared with 10 assays with the membrane immunosensor).

The use of pAPP as the substrate for EC enzyme immunoassay was demonstrated above[46] in a competitive ELISA for theophylline. IgG and AFP also were used by the same authors to demonstrate good detection limits achievable by using EC methods for detection in a sandwich heterogeneous assay. Under these conditions, the detection limit for IgG is 5.4 amol ml^{-1}, with a linear range up to 10^5 times the detection limit. In the sandwich heterogeneous assay for AFP at the femtomole level, the detection limit was 0.16 μg l^{-1}, with the lowest measured AFP being 0.32 μg l^{-1}, with a CV of 4.2%. The detection limit obtained is determined by the value of the zero dose response and not by the ability to detect pAP.

b. Chemiluminescent Detection

The use of CL labels in immunoassays has become increasingly popular in recent years[71–74] because of the high sensitivity and wide dynamic range afforded by this method. Three types of chemiluminescent compounds are acyl hydrazides (luminol), acridinium esters, and trichlorophenyl oxalate esters. The oxidation of luminol and its derivatives by hydrogen peroxide in the presence of a catalyst (e.g., HRP) has received considerable attention. Recently, certain phenol derivatives that enhance the intensity and prolong the chemiluminescent reaction have been reported.[75]

Maeda and Tsuji[63] applied the same FIA-CL system described above to the determination of AFP by a sandwich ELISA. The calibration graph is linear over the range 2.5 to 50 ng ml^{-1}, with a detection limit of 2.5 ng ml^{-1}. The sensitivity of the proposed method is higher than that of other enzyme immunoassays for AFP.

Shellum and Gubitz[76] also reported a FIA system for carrying out a sandwich immunoassay. A Teflon® tube containing antibodies covalently immobilized to a rigid beaded support is used as the immunoreactor. Buffer is constantly pumped through the immunoreactor and the assay is conducted by simply injecting sample and reagents into the buffer stream. The system uses acridinium ester-based CL

for detection, which has the advantages of simplicity and sensitivity. The determination of mouse IgG was chosen as a test system because of the potential analytical applications in MAb production and because of the fact that anti-mouse IgG immunosorbent can be used as a universal solid phase for MAb-based immunoassays. A two-site immunoassay is accomplished by consecutive injection of the sample, acridinium ester-labeled antibodies, and alkaline hydrogen peroxide to initiate CL. The light emission is collected directly from the transparent immunoreactor, which is then regenerated in preparation for the next sample injection. The detection limit, sensitivity, and time required per sample are dependent on the assay flow rate; for times of 10, 12, and 18 min per sample, the limits of detection are 500, 200, and 50 amol of mouse IgG, respectively. The precision of replicate measurements has a RSD of 3 to 4%, and the same 20-μl immunoreactor can be used for at least 1 week with an inter-assay RSD of 5.9%

A FIIA for human IgG using stopped-flow and CL detection of HRP products was reported by Osipov et al.[77] The kinetics of the immobilized antigen-antibody interaction was studied and the quantitative time-concentration ranges were estimated. Both immunochemical steps were pursued in the kinetic regime. The enhanced luminescent reaction using luminol and *p*-iodophenol as substrates was used to detect the peroxidase label. A Tecator FIA 5020 instrument equipped with two pumps and a double-injection valve was used and the conditions for CL reaction were optimized. The detection limit for peroxidase in a 3-min assay was 5.10^{-16} mol per tube. The detection limit for IgG was 10^{-9} *M*, the overall time of the assay being 5 to 10 min. However, there are some disadvantages associated with using packed tubing as immunoreactors for the solid-phase FIA procedure: the light scattering from the packing material, which seriously compromises the detection of CL, the small diameter tubing of the FIA manifold, which often cannot be packed uniformly, and the difficulty in packing the tubing consistently every time, resulting in significant variation, particularly in sensitivity.

Finally, Liu et al.[78] reported a study of a FI solid-phase chemiluminescent sandwich immunoassay system, using a thin-layer flow cell as the immunoreactor with antigen (bovine IgG) immobilized on a membrane. The membrane was a polyvinylidene difluoride polymer. The analyte in the form of mouse monoclonal anti-bovine IgG was injected into the flowing stream, followed by a goat anti-mouse IgG HRP conjugate. The HRP was used to catalyze the enhanced luminol reaction, resulting in CL, which was directly detected within the immunoreactor. A detection limit of 1 fmol was obtained, while spiked serum gave a precision of 8.7%. Although in the original paper this assay is referred to as a "sandwich", it could be more properly classified as a *heterogeneous noncompetitive EIA* because the term "sandwich" is often reserved for assays in which the analyte (antigen) is entrapped between primary antibody and enzyme-labeled antibody.

c. Fluorimetric Detection

In previously mentioned papers,[47–49] Stöcklein et al. described an on-line detection system for MAb production based on FIIA. In addition to the use of a competitive assay, they also performed a sandwich ELISA, with the product of the enzymatic indicator reaction being measured fluorimetrically.

For the application of the sandwich assay in FIA, higher concentrations of antibody for immobilization and conjugate were necessary than in the competitive assay, in order to obtain a correlation between antigen concentration and fluorescence. Therefore, their concentrations were doubled. Furthermore, the incubation time for the antigen and conjugate was increased to 5 min each, with a total cycle time of 25 min per assay. The detection limit obtained by the sandwich FIIA was 1 μg ml^{-1} antigen, and the dynamic range in the sandwich assay (1 to 10 μg ml^{-1} antigen) was limited mainly by the amount of immobilized antibody. However, in the competitive assay, a broader range of antigen could be measured with lower concentrations of immunoreagents. Hence, for this type of FIIA, including support exchange after each assay, the competitive assay is preferred. Control experiments were performed for each assay system using membranes or magnetic particles treated with caseine alone. There was no significant change in fluorescence within the antigen range applied. The automation of

the FIIA described has not been achieved yet, but a membrane exchange and magnetic particles dosage is being developed.

Finally, Stöcklein and Schmid[50] discussed the advantages and limitations of the sandwich assay for mouse IgG. With this format, an increase of incubation time from 30 min to 4 h is necessary. The advantages of a competitive FIIA compared with the sandwich FIIA are short analysis time, fewer incubation steps, and lower cost. The advantages of the sandwich FIIA are simpler data processing and less dependence on the analyte subclass and variability of the sample size.

D. Liposome-Based Techniques

Other systems based on heterogeneous immunoassays have been suggested. Among them, those using liposomes seem to be quite promising. When an analytical sample containing the compound of interest is injected into the FIIA system, interactions between analyte and its antibody occur on the surface of the immunoreactor. Detection of this interaction can be mediated through the use of liposomes. Liposomes are spherical membrane structures that form spontaneously when phospholipid molecules are dispersed in water. The bilayer membrane of liposomes is similar to cellular membranes and surrounds an entrapped aqueous volume. Water-soluble fluorescent molecules, such as the dye carboxyfluorescein, may be trapped inside the liposomes, and untrapped dye molecules are separated from the liposomes by gel filtration. These structures have great potential for application in drug delivery and also are attractive for use in diagnostic and other assay systems, where they provide signal enhancement due to the many molecules that can be released for detection.

Liposomes may be "sensitized" to a particular antigen through covalent binding of that antigen to the polar head groups of the phospholipid molecules. When the liposome is formed, part of the antigen remains exposed to the external solution and is available for interaction with antibody binding sites. Generally, phospholipid molecules derivatized with the antigen are inserted into the membrane of each liposome. The antigen attached to the liposome can compete with free antigen (analyte) molecules in solution for binding sites on the antibodies covalently bound to a solid support. Liposomes that do not bind to antibody sites in the reactor are carried downstream to a post-column mixing chamber where a surfactant is added. Surfactants disrupt the membrane of liposomes, releasing the dye that passes into the fluorescence detector. The reactor column is then regenerated by disrupting the interaction between the antigen and the immobilized antibody, and a new sample can be injected. Alternatively, the liposomes displaced from the beads during the reactor regeneration process can be lysed by surfactant and the released dye measured in the detector. The advantage of the second approach is that liposomes are now free of sample matrix effects. In this operation mode, the analytical signal produced is inversely proportional to the analyte concentration.

The detection and signal amplification using liposome labels are not dependent on a secondary reaction, as is required for enzyme labels. Therefore, the use of liposomes has the advantage of providing an immediate large signal amplification. The objective is to use liposomes containing a compound as analytical tools for signal enhancement in immunochemical-based FIA systems. Successful application of liposomes in an unbiased competitive assay in FIIA requires that the liposomes and solution species have identical and reproducible flow behavior so that liposomes and soluble analytes are uniformly mixed within the reaction volume.

Locascio-Brown et al.[79] examined the hydrodynamic behavior of liposomes to evaluate their usefulness as analytical reagents in continuous flow systems. Liposomes were found to be unstable when exposed to underivatized glass bead columns due to the loss of lipid from the liposome bilayer to hydrophobic sites on the column. Conditioning of the system with lipid prior to the introduction of liposomes helped to stabilize the structures by saturating all exposed hydrophobic sites. On the other hand, liposomes, when exposed to a properly derivatized glass, are stable in the flow system and do not release their trapped contents, even at flow rates of 2 ml min^{-1}.

1. Fluorimetric Detection

Locascio-Brown and co-workers[80–82] developed a novel FIA system that contains an immunospecific reactor column and utilizes liposomes for detection. The fluorophore-loaded liposomes used in this assay can provide signal enhancement in the range of 1000 to 1 million times per binding event, making fluorescence assays competitive in sensitivity with radioimmunoassays. The flow injection system contains a glass reaction column packed with nonporous glass beads that provides the solid phase for the immunospecific reaction. The antibody is covalently bound to the solid support in a manner that ensures the proper orientation of the antibody binding sites so that there is very little loss in activity. Free antigen and liposome-conjugated antigen are injected together and compete for binding with the immobilized antibody. Bound and/or unbound liposomes are lysed, and the fluorescent dye released and measured in the detector.

More recently, the same group[83] developed two different FIIA applications using fluorescent dye-containing liposomes: the quantitation of the therapeutic drug theophylline and that of anti-theophylline antibodies. For both assays, anti-theophylline is immobilized onto nonporous silica particles that are packed into an immunoreactor column, and both assays employ liposomes that contain theophylline-phosphatidylethanolamine in their membranes. For the detection of anti-theophylline, the analyte competes with immobilized anti-theophylline for sites on the theophylline-labeled liposomes. In the detection of theophylline, the analyte competes with theophylline-labeled liposomes for immobilized antibody sites. The results in both cases is an inverse relationship between the amount of analyte in the sample and the amount of liposomes that bound to the solid support. The difference in the sensitivity found (ca. 100 times) is a function of the assay format and the characteristics of the interaction of liposomes with immobilized antibody. Potassium isothiocyanate and 1-*O*-octyl-β-D- glucopyranoside were compared for their effectiveness as column regeneration reagents, on the basis of three criteria: regeneration of antibody binding sites, long-term effect on antibody activity, and effect on fluorescence signal. Under the assay conditions, 1-*O*-octyl-β-D-glucopyranoside was found to be preferable.

Using the same theophylline–anti-theophylline system, a model addressing the competitive binding under equilibrium conditions also was reported.[84,85] In this FIIA system, equilibrium in the immunoreactor could be achieved easily by making the residence time of the sample in the immuno-reactor long enough. The model aids in the optimization of the sensitivity and dynamic range by evaluating the effects of such parameters as concentrations of liposomes and immobilized antibodies, binding constants of liposomes and haptens, and steric considerations associated with the relatively bulky liposome reagent. The system can be calibrated and is reusable, and automated sequential analyses can be performed at room temperature with picomole sensitivity and a day-to-day CV of less than 5%.

2. Electrochemical Detection

A double-amplification immunoassay for theophylline, based on the use of peroxidase-loaded, theophylline-conjugated liposomes and a packed-bed antibody reactor column in an automated FIA system has been developed.[86,87] The double amplification is achieved by means of liposome-encapsulated peroxidase enzyme molecules that are released subsequent to a competitive immunological reaction. The released peroxidase enzymatically cleaves (from an organofluorine substrate) fluoride ions that are then measured potentiometrically. The competition between the analyte molecules and theophylline-derivatized liposomes for immobilized antibody sites in flow-through immunoreactor columns results in unbound liposomes being carried downstream where they are ruptured in the presence of the H_2O_2 and *p*-fluorophenol. The immunoreactor column is then regenerated with a chaiotropic agent, and the next sample or calibration can be run. By means of this method, theophylline can be determined over a range of concentrations from 0.2 to 4000 ng ml^{-1}. The detection limit of 200 pg ml^{-1} corresponds to about 100 fmol of theophylline measured in the 100-µl sample injected, and this is equivalent to that reported for other methods (e.g., RIA).

Finally, an immunoassay technique to measure anti-asialo-GM1 (GA1) antibodies using multilamellar liposomes was described by Katoaka et al.[88]

In this case, the liposomes are composed of dipalmitoylphosphatidylcholine, cholesterol, and GA1 antigen, and entrap molybdate as marker ions. Molybdate is released by a complement-mediated immunoreaction, and acts as a catalyst for promoting the H_2O_2-iodide ion redox reaction. After mixing and incubating antibody sample, liposomes, and complement, the resulting solution is injected into the kinetic-FIA system. The decrease in the number of iodide ions by the molybdate ion-catalyzed reaction is monitored using an iodide ion-selective electrode. The method can be used to detect as low as 10^3 to 10^4 dilution of anti-GA1.

III. RECENT DEVELOPMENTS

Interesting areas for future development in the use of FIA to study biochemical selective interactions include the wider use of immobilized reagents, the application of dynamic detection systems, the general availability of MAb, and advances in fully automated instrument design. FIA in life sciences is still open to further improvements. Some of the targets to tackle are miniaturization and simplification of the FIA-based immunoassays. In parallel, the rapid developments in immunoassays over the last 2 decades have been directed toward increasing the sensitivity and degree of automation of the various techniques. In fact, there are several different lines of research being pursued at present that will contribute to the development of immunoassay technology and usefulness. One of these is the development of alternative assay methodologies or technologies. The future of immunoassays lies with improved tools that will provide simpler means by which to carry out assays. The development of MAb has provided the ultimate in specificity for antibody-antigen interactions and they are already being used for these purposes.

In recent years, several pesticide-specific MAbs have been developed. Samarajeewa et al.[89] described a FIIA format that allows detection of single triazine pesticides at a level of 0.1 $\mu g\ l^{-1}$ and below. At present, the authors are attempting to automate the procedure through the use of a membrane reactor developed for this purpose. The principle consists of carrying out the immunoreaction in a miniaturized reaction chamber in which the antibody is immobilized on a continuous band of membrane, whereby the waste immunocomplex can be automatically eliminated from the flow system.

A number of different ways of measuring the antigen-antibody interaction have already been described. Among them, much research effort has been directed recently toward developing biosensors or immunosensing techniques. A biosensor can be defined as a device that integrates a biological component (e.g., enzymes, antibodies, or cell receptors) intimately to a transducer (e.g., electrochemical, optical, piezoelectric, or thermistor solid-state devices). Whenever an interaction occurs between the target analyte and the biological component of the biosensor, some property of the transducer is altered and subsequently measured.[90] The instrumentation associated with the sensor must be robust, easy to use and low-cost, and must have low maintenance and calibration requirements.

Immunosensors are biosensors that exploit the immunological binding event. Generally, these sensors have competitive binding principles and are based on immobilizing the binding reagent on the surface of an electrochemical, piezoelectric, or optical detector. Immunosensors have been applied successfully in research work and the advantage of their easy use as well as their sensitivity and specificity makes them comparable to conventional immunoassay techniques.[91]

Some new trends in the field of optical immunosensing include the application of internal reflection spectroscopy, surface plasmon resonance (SPR), and evanescent wave. Following are the more recent applications of immunosensors in the flow system. The kinetics of the antigen-antibody interactions can be followed directly without labeling using SPR, whose response is sensitive to changes in the refractive index in the probed volume. An integrated fluid-handling system for multichannel biomolecular interaction analysis was described by Sjolander and Urbaniczky.[92] The measurement of changes in the angular position where SPR occurs at a biospecific active surface permits monitoring of the reaction between biological molecules in real time. The sample zone dispersion is minimized by the low dead volume in the system, accomplished by using integrated sample loops and thin conduits. The sensor chip with the biospecific active surface is reusable and easily exchangeable.

The results obtained using a theophylline MAb as the word analyte compared with a theoretical model. The RSD is 5% and the assay time is 10 min. The detection limit is approximately 10 pg of analyte on the probed spot of the surface. Possible improvements in sensitivity and detection limits are discussed.

Recent investigations using a FIIA system[93] have shown the possibility of quantitative regeneration of a surface-immobilized antibody in an affinity column. Choquette et al.[94] adapted this approach to optical waveguide sensors and developed an immunosensor for theophylline in a competitive assay using antigen-tagged liposomes. The regeneration of immunosensors is accomplished under flow conditions using a moderate affinity antibody, and multiple analyses can be performed with a single waveguide sensor. Sensors capable of more than 15 sequential measurements have demonstrated better than 10% precision. The use of theophylline-labeled liposomes in this competitive immunoassay provides one order of magnitude signal enhancement over theophylline derivatized with fluorescein.

IV. CONCLUSIONS

The recent and rapid growth in immunochemical methods can be attributed partly to the availability of polyclonal and monoclonal antibodies for a variety of compounds of great significance in many areas.

Flow injection based on antibodies provides a powerful tool for the development of analytical protocols for semicontinuous operation and/or the analysis of a large number of samples, and could have a significant value in routine analysis.

FIIA is beginning to gain acceptance by Public Analyst and Quality Controllers and offers the easiest, fastest, most accurate, and cheapest methods currently available. The application of FIIA techniques can contribute enormously to quality control in different areas such as clinical, agricultural, food, environment, and fermentation processes. Future immunochemical techniques such as recombinant antibodies, catalytic antibodies, etc. could be applied to a variety of analytical problems, for example, multiresidue immunoassays, detection of organometallic compounds, etc., using flow injection methodologies. Additionally, the development of immunosensors and their application in FIA systems also could offer the capability of performing continuous on-line monitoring.

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