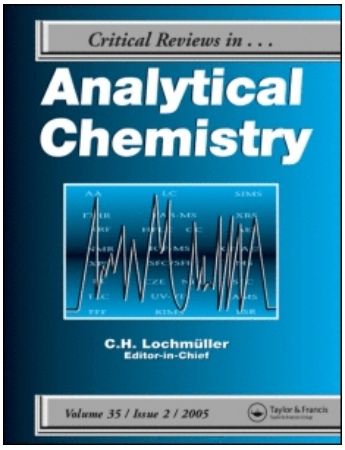

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Recent Developments in Flow Injection Immunoanalysis

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ABSTRACT: Flow injection coupled to immunoassays (FIIA) is a powerful tool for the development of analytical protocols and has been applied in fields other than clinical. This article presents new FIIA contributions in areas such as biology, environmental, bioprocess monitoring for on-line applications or drugs. The development of FIIA methods using different labels, liposomes, immunoreagents, or immunosensors is reviewed. Finally, new trends in FIIA and the feasibility of using other techniques, such as high-performance immunoaffinity chromatography (HPIC) or capillary electrophoresis (CE) are also taken into account.

KEY WORDS: flow injection immunoanalysis, immunoassay, drugs, plaguicides, bioprocess monitoring.

I. INTRODUCTION

Immunoassays (IA) are analytical methods that are based on a reaction between a target analyte and a specific antibody (Ab) that are widely used in biochemistry and in the future could play a key role in the laboratory. The introduction of IA into other fields is ensuring the development of other formats that are applicable to biotechnology, environmental, process control, etc.

With the movement toward automation in IAs, more and more research is being done with continuous-flow analytical techniques, especially flow-injection (FI). With respect to immunoassays, FI offers precise control of reaction times, compatibility with any heterogeneous or homogeneous format and detector, reuse of supports and reagents, improvement in accuracy and precision with a sensitivity similar to batch methods, dramatic reduction of analysis time close to real time performances, flexibility, on-line coupling to industrial processes, easy calibration and recalibration, the possibility of integration of reaction, separation, and detection processes used with different formats,[1,2] use of kinetic and differential measurements, and application as routine methods and screening.

Flow injection coupled to immunoassays (FIIA) is arising as a powerful tool for the development of analytical protocols, and the application of FI techniques is expected to enormously contribute to improving the speed and quality of the immunoassay.

Several studies on this subject have been reported.[3,4] Puchades et al.[5] give an overview on the applications of flow injection techniques in immunoanalysis as well as examples of various IA formats commonly employed to visualize the primary antibody-antigen (Ab-Ag) reaction. In this section, the new FIIA applications that have appeared in

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the last 2 years are reviewed and the potential merits of their use are discussed.

The recent rapid growth in immunochemical methods is partly attributed to this availability of policlonal antibodies (P_{Abs}) and monoclonal antibodies (M_{Abs}) for a variety of compounds and to the widespread use of Ab obtaining and purification techniques.

Crude isolated immunoglobulin G (IgG) contains 0.5 to 2% of the specific Ab.[6] However, in order to reduce the amount of Abs consumed, and in view of the applications in isolating residual amounts of analyte, the capacities obtained are suitable. Higher capacities can also be obtained using M_{Abs}. The main advantage of the use of M_{Abs} is the possibility of having an unlimited amount of the specific Ab.

Monoclonal Abs generally possess higher specificity than policlonal Abs, which are composed of an ensemble of different protein bodies. It remains unclear whether the use of M_{Abs} or P_{Abs} is more advantageous. With respect to FIIA methodology, M_{Abs} seem to be more interesting, especially if they are haptens. The use of a sole clon is easier and theoretically simplifies the design of methods concerning affinity, selection of immobilization and labeling reactions, lesser carryover, etc.

Whether monoclonal or policlonal Abs are selected depends on the type of analysis, the available media, and the use requested. For small-scale laboratories, P_{Abs} production is the direct way to get quick results. In the literature reviewed, there is a clear trend to use M_{Abs}. For worldwide marketing, M_{Abs} are necessary.

An IA can be homogeneous or heterogeneous; both have been developed in FIIA. The typical method for homogeneous assays involve monitoring of a change in the characteristics of the Ag-Ab complex.[7] Its advantage is that no separation step is required; however, this also has interference problems from the sample matrix. There are a number of homogeneous flow injection assays,[3,8,9] including a prototype of a commercial system.

Although homogeneous assays are very interesting, heterogeneous assays comprise a majority of currently performed IAs, because the choice of homogeneous assays is limited by the restrictive requirement that some change in the signal must occur after binding. The use of a solid phase eliminates this requirement by physically separating the bound and the unbound portion of the analyte, typically by means of an appropriate Ab immobilized on the surface of a solid phase. In this way, the analyte is selectively bound to the immobilized Ab and the remaining portion of the sample can be removed in the washing steps.

FIIA heterogeneous assays most often carry out the separation step using an packed-bed reactor that binds the species of interest while allowing the rest of the matrix to pass through the reactor.

Automatized heterogeneous IA offers many advantages.[10] They are more sensitive, especially for large proteins, less prone to interferences, and are extremely flexible to the point of choosing the detection principle and solid phase. Heterogeneous flow injection immunoassays offer accelerated binding kinetics. First, there is a very high surface area-to-volume ratio in the immunoreactor, and the effective concentration of capture antibody can be extremely high. On the other hand, the binding reactions do not have to rely on passive diffusion to bring reactants together. The flowing stream actively brings the sample in contact with the solid-phase antibody. These factors result in a greatly enhanced antigen-antibody (Ag-Ab) encounter rate in nearly quantitative binding during the sample's short residence time (min) in the immunoreactor.

Continuous heterogeneous flow methods offer other advantages for automatic IA,[11] namely, the many washing steps required are inherent in a flowing system and the

immunoreactor acts to concentrate the sample.

The solid phase is usually made by coupling antigens or antibodies to preactivated particles, although activated membranes, capillaries, or other high surface area matrices can also be used. The first step is the selection of the support and the immobilization of the antibody (or antigen). The choice of support to which the Abs or Ags are immobilized is of central importance. Nonspecific adsorption must be kept to a minimum as any irrelevant binding to the support will influence the background value and the sensitivity of the assay. The immobilization of the antigen has several advantages over the immobilization of Abs.[12] Despite this, most works immobilize Ab, because general and widespread immobilization procedures are used. On the other hand, the immobilization of low-molecular-weight analytes requires specific reactions as well as an adequate orientation.

There are several types of solid phases used for heterogeneous IAs. Large-diameter beads are used because they can be easily washed and retained in a test tube during an analysis. Immunomagnetic beads can be held in a magnetic field to facilitate washing and separation steps or repositioning of beads. This type of support, as well as exchangeable membranes,[13] have the advantage that no regeneration steps are necessary, thus reducing significantly the time of analyis. However, the expected performances in flow methods have not been obtained due to the imprecision of the results and the need of operating with complex mechanisms.

A variety of supports are available, such as soft gels (agarose), cellulose, resins, polymers, bonded-phase silica, and glass beads, in different presentations. Methods to immobilize antibodies onto various stationary supports are well known. No single system seems to work best for all antibodies, and the immobilization of antibodies is still a trial and error procedure. Two critical parameters to study are the loading of the antibody on the support and the binding activity of the Ab. These criteria are important because they influence the sensitivity and the selectivity of the immunoaffinity technique.

However, a common drawback of many heterogeneous flow-based methods is the need of using columns packed with Ab-coated beads, which serves as retention surface. This retention surface cannot be discarded after each use, but must be regenerated prior to the next assay. Regeneration requires breaking the Ab bond to the analyte without altering in any way the immobilized Ab, because denaturation will result in different binding characteristics and ultimately erroneous results. The use of chaiotropic reagents to dissociate the Ag-Ab complex has often resulted in a significant loss of Ab activity. Therefore, the dissociation step in which the affinity sorbent is rinsed and restored for the next cycle is a critical point. It is important to achieve the most complete dissociation as possible, because any remaining reagents will reduce the capacity and activity of the column. The higher dissociation rates exhibited by moderate-affinity Abs (K_a $<10^8$) make the regeneration of the Ab feasible from the thermodynamical point of view using mass action in a flow system. The dissociation step is also time-dependent and the kinetics of Ab-Ag complex binding to solid-phase depends on the diffusion rate and steric interactions. A compromise must be reached between the amount of time consumed and the amount of undissociated material remaining on the support.

Several techniques have been proposed to yield useful information from Ab-Ag binding in FI systems. The disadvantages associated with the use of radioactive materials have led to the implementation of a wide range of new labels, first by enzymatic methods and then fluorescent, chemiluminescent, and infrared active.

The use of a given marker depends on the format chosen and the required sensitiv-

ity. Generally, the sensibility obtained with the flow methods is similar to that of the batch techniques (e.g., ELISA). However, from a practical point of view, the amounts to be detected are minimal, and low detection limits must be reached, either by law or to advantageously compete with other established techniques.

The enzymatic markers with fluorescent detection are the best ones. This type of label is straightforward with high selectivity, good performance on immobilization, amplification of the obtained signals by means of stop-flow, and the specificity and selectivity of the fluorescent detection.

Fluorophores, which are commonly used in fluoroimmunoassays such as fluorescein isothiocyanate (FITC) or Rhodamine G, exhibit a small Stokes shift and therefore lack complete separation between excitation and emission wavelengths. Because fluorescent Eu(III)-chelates show a Stokes shift larger than 270 nm, the alternative of using this special class of markers is starting, especially in clinical analysis and also in environmental analysis.

A new macrocyclic ligand for Eu(III) was synthetized and its fluorescence characteristics examined. The complex of the ligand Eu(III) had a higher fluorescence intensity and a longer fluorescence lifetime, and has been used as label in time-resolved fluoroimmunoassays.[14,15]

A study of several fluorescent dyes as labels for the developing of a FIIA was carried out by Palmer and Miller.[16] Fluorescence emission and absorption are reported for Nile blue, toluidine blue, methylene blue, oxazine 750, oxazine 4, Azure A, Azure B, and cresyl violet. The best flow injection profile was obtained using Nile blue conjugate and a Sepharose immunoreactor.

Recent interest in acridinium ester chemiluminescence (CL) has focused on its application to immunoassay. The use of acridinium esters as CL labels for Abs and other molecules seeks to exploit the characteristically low detection limits and wide dynamic ranges associated with the reaction. Wilson and co-workers[17] have demonstrated that flow injection used in association with CL immunoassay offers the advantages of greater speed and reproducibility and should facilitate the easier automation of complicated assays.

Litting and Nieman[18] have been investigating analytical advantages that arise from electrogeneration of CL emission from several systems. Acridinium ester is traditionally triggered by the addition of a solution of H_2O_2. The electrochemical generation of the hydrogen peroxide necessary for CL reaction within the observation cell eliminates the need for the addition of unstable peroxidase solutions and additional solution streams. Hydrogen peroxyde was generated at a vitreous carbon cathode and chemiluminescence was measured with a photo-multiplier. Calibration graphs were rectilinear for 0.01 to 100 p*M*-phenyl acridinium-9-carboxylate. On the other hand, the intensity of the CL signal from an aqueous peroxyoxalate CL reaction can be significantly enhanced in the presence of various proteins, including Abs. An FI system using enhanced peroxyoxalate CL was developed by DeLavallle and Grayeski[19] for the determination of various proteins such as albumin and IgG in human serum to improve precision and to automatize these measurements. Recently, Katayama et al.[20] reported aryl oxalate CL as one of the most sensitive methods for the determination of hydrogen peroxide and Abs derivatized by glucose or β-galactosidase.

IA with electrochemical detection (EC) is more widely used than spectrophotometric techniques. It is relatively inexpensive, easy to operate, and may be used to make measurements in turbid media. Several studies have been carried out in order to amplify the response of a IA using EC detection.[20–24] All of them used alkaline phosphatase as the enzyme label. The IgG level was evaluated by detecting the concentration of PAP, which

is the product of the enzymatic reaction of the substrate PAPP.

The manner for enhancing detection is different in each case. For example, Niwa et al.[23] use an interdigitated array microelectrode for PAP determination and Thompson et al.[24] used a good combination of buffer concentration, pH, and additives to raise the activity of alkaline phosphatase.

The use of liposomes provides an immediate large signal amplification and is used for signal enhancement in immunochemical-based FI techniques.

Previous studies have demonstrated the advantages of liposome-encapsulated dyes rather than enzymatically produced color to enhance the signal obtained in the competitive reaction, and they have used this amplification technique in an automated FIIA for a environmental laboratory.[25,26] Liposomes have been used more often in homogeneous-phase assays requiring the lysis of immunospecific liposomes and also as reagents in solid-phase assays.

Plant et al.[27] presented a dynamic model to characterize multivalent binding as the continuum limit of a one-step stochastic process using a FIIA. Assumptions for the theory are based on data in which liposomes were used as a model system for multivalency. Locascio-Brown and co-workers[28-30] have made very interesting contributions in the study of the usefulness of liposomes as analytical reagents in continuous flow systems as well as in the performance of the liposomes themselves.

There is a continuing effort to develop new immunoanalytical procedures, particularly in the field of the organometallic tracers. Organometallic labels provide many analytical methods suitable for the detection and quantitative determination of the label by spectrophotometric and electrochemical techniques.[31]

A sensitive EC determination of cobaltocerium-labeled amphetamine illustrates the potential of using an electrode coated with an anionic polymer (Nafion) with a cationic redox-labeled hapten.[8]

Molibdate ions entrapped in liposomes as marker ions were used by Kataoka et al.[32] These metals acted as a catalyst for promoting the hydrogen peroxide-iodide redox reaction. The decrease in the number of iodide ions by the molibdate ion-catalyzed reaction was monitored using an iodide ion-selective electrode. The marker ion release was specific for the antibody used in the immunoreaction.

Recently, studies based on new dyes that can be used as labels in immunoassay such as near-infrared dyes[16] or new detection systems such as near-infrared fluorescence[33] have been reported. In the literature reviewed, the markers used in flow in the differents formats are nearly always fluorescent (fluorescein) or enzymatics (alkaline phosphatase, glucosidase, horseradish peroxidase).

The application of FIIA in clinical and biomedical analysis, environmental analysis, bioprocess control, and miscellaneous aspects of FIIA are discussed.

A. Clinical and Biological Analysis

In the following paragraphs, we discuss the current status and trends of flow injection immunoassay techniques for clinical and biomedical analysis.

Electrochemical detection, because it is fairly sensitive, adaptable to very small samples, and generally very well suited to FIIAs, is the most common detection system used in this field.[9,34-37] The immunoassay is performed in batch and FI is only needed in the detection steps.

In most of the reported electrochemical EIA systems,[34-36] *p*-aminophenyl phosphate (PAPP) was used as the substrate of alkaline phosphatase and hydrolyzed to *p*-aminophenol (PAP). The amount of PAP produced from the assay was proportional to the amount of the analyte in the sample. In all cases, the

detection of PAP was done electrochemically by means of FI systems.

Human IgG was measured over a linear range from 0.1 ng/ml to 1 mg/ml using an ELISA microtiter plate, based on the principle of a sandwich ELISA and electrochemical measurings of PAP by an automated FI system.[34] The results indicated that this approach had advantages in sensitivity and measuring range.

A competitive ELISA with EC detection in an FI system has been developed by Kronkvist et al.[35] for the determination of the steroid drug budesonide. Plasma samples were cleaned from interfering and cross-reacting compounds by two treatment steps consisting of a solid-phase extraction and a liquid chromatography fractionation. The detection of PAP was carried out electrochemically using a glassy carbon electrode at 250 mV. Budesonide could be quantified down to 10 p*M*. The major sensitivity-limiting factor was the amperometric background response, probably due to spontaneous hydrolysis of PAPP to PAP.

Yu et al.[36] developed a sensitive heterogeneous EIA for thyroid-stimulating hormone (TSH) by modifying a commercial available two-site immunoenzymometric assay. The detection of the final product (PAP) was done by oxidative amperometry in an FI system using a glassy carbon electrode at 325 mV. The linearity range was 50.0 fmol to 100 pmol PAP, with a detection limit of 10.9 fmol PAP.

A flow injection amperometric system with a Pt-wire working electrode and an Ag-AgCl counter-electrode was developed for monitoring the results of enzyme immunoassay.[37] The possibility of the optimization of the geometry and hydrodynamic properties of amperometric detectors in flow systems was demonstrated. The system was tested in the determination of peroxidase and immunoglobulin E in blood serum, with a sample throughput of 400 per hour.

An electrochemical homogeneous EIA for theophylline was performed by Yao et al.[9] in whole-blood samples with no separation steps for the blood cells. The assay used a Syva EMIT Theophylline Kit. One of the enzymatic reaction products, NADH, reduced 2,6-dichloroindophenol (DCIP) to $DCIPH_2$, which was detected EC by flow injection analysis. Recoveries ranging 92 to 111% were obtained and the linear range extended from 2.5 to 40 mg/l.

Fluorimetry has also been applied to FIIA as a method of detection in the clinical field. A protein immunoreactor was used for on-line fluoroimmunoassay of human transferrin.[38] Antibody immobilized on protein A and antibody-antigen complex formation took place in phosphate-buffer saline. After washing off excess Lucifer yellow VS labeled human transferrin, the Ab-Ag complex was eluted with acid buffer and detected.

A novel flow injection-immunometric assay (FIIMA) has been proposed by Miyairi et al.[39] for the determination of progesterone with high specifity and simplicity. Employing a reactor consisting of labeled fluorescein isothiocyanate (FITC) antibody bound on immobilized Ag, the bioactive material bound with labeled Ab in an eluent from the reactor can be determined sucessively. In this method policlonal Ab is applicable without any prior purification.

The continuous flow analysis using immobilized Ag to remove unbound labeled Ab after the competitive reaction with Ag has also been developed, but a highly purified monospecific Ab such as M_{Ab} is required to minimize the background level.[40,41]

Micromachining of silicon or other planar glass plates provides a way to develop miniaturized chemical analysis devices.[42,43] Son et al.[44] developed a rapid and multisample analysis that was an application of micromachining techniques coupled with an electric-field-enhanced latex immunoreaction. This research has been done as a major part of the development of a fully automatic flow injection system for α-ferroprotein in serum using fluorescence

image analysis. The lowest detectable amount of α-ferroprotein was 10 pg/ml, which is more sensitive than previous research works (500 pg/ml).[45] The linearity was obtained from 10 to 500 pg/ml α-ferroprotein.

On the other hand, two research groups headed by Locascio-Brown and Palmer work on the development of new flow injection competitive IA for clinically interesting species, using different detection systems, generally fluorescence.

Using liposome signal amplification and fluorescent detection, the Locascio-Brown research group developed two FIIA for theopylline[28,29] and another one for the 17-β-estradiol hormone.[30] Choquette et al.[28] developed a regulable planar waveguide immunosensor for theophylline using antigen-tagged liposomes. The aqueous inner part of liposomes is filled with a solution of carboxyfluorescein, and a theopylline lipid tag is incorporated into the outer membrane. The maximum number of sequential injections achieved without serious degradation in the activity is approximately 15, with a precision better than 10%. The use of theophylline-labeled liposomes provides 1 order of magnitude greater signal enhancement over theophylline derivatized with fluorescein.

With the same immunosystem (theophylline-labeled liposomes containing carboxyfluorescein), Locascio-Brown et al.[29] developed an FIIA for theophylline using a silica solid-phase reactor column containing immobilized Ab. The calibration graph was linear from 0.025 to 0.4 mg/l. Effective regeneration of the immobilized Ab (phosphate buffer saline, pH 7.4) allows the reactor to be reused for hundreds of sequential samples. The total analysis time for each sample is 16 min. The same amount of time is required for measuring estrogens in an FI system incorporating a column-type reactor packed with a solid silica particles with immobilized antigen 17-β-estradiol.[30] Anti-estradiol is noncovalently conjugated to the liposome through a streptavidin-biotin linkage. A 4-cm column is enough to achieve detection of 17-β-estradiol in serum.

Plant and co-workers use fluorimetric detection instead of liposomes as signal enhancement in all the heterogeneous competitive FIIAs described. This group immobilizes the Ab via protein A. This immobilization mode is poorly used in FIIA despite of their potential advantages.[46,47]

A heterogeneous on-line electrochemical enzyme immunoassay has been described for the antiasthmatic drug theophylline.[47] In this assay the antibody, label (alkaline phosphatase), and sample are sequentially immobilized onto the controlled pore glass (CPG)-Protein A immunoreactor. PAP has been used as a detection system with a detection limit of 25 ng/ml. The calibration range was linear between 60 to 300 ng/ml of theophylline. The total time for this on-line immunoassay for theophylline was 10 min compared with 18 min for the previously developed system.[48] Other fluorophores (Nile blue) and supports (sepharose) are being investigated for theophylline.[16]

The same FIIA format[47] was used for the on-line fluorescence determination of trasferrin levels in human serum.[49] Lucifer yellow VS was the fluorophore. The calibration graph was linear from 25 (detection limit) to 600 μg/ml of transferrin. The immunoreactor needed replacing after 60 runs.

Protein G, which is similar to protein A, was utilized by Khohkar et al.[50] for insulin, using the heterogeneous fluorescence IA described previously.[49] Insulin concentrations of 50 pg/ml could be determined with good repeatability and reproducibility (rsd = 4%) with a sampling frequency of approximately four per hour.

A rapid fluorescence FIIA has been developed by Palmer et al.[51] for serum tesosterone using a novel perfusion chromatographic matrix coated with protein A (POROS). The fluorophore used was Texas Red. For each assay the Ab-protein A reaction takes place at nearly neutral pH, and the

complexes are eluted at acidic pH. Both preincubation and on-line assay formats were investigated, and the use of avidin-biotin as the spacer arm is also described. The total time for the preincubation and on-line assays was less than 4 and 6 min, respectively. Some work carried out recently using this matrix (POROS II A) have shown that the preincubation and on-line incubation assay times can be reduced to 2.5 and 1.5 min, respectively, with sample throughputs of approximately 25 to 40 h^{-1} and a column lifetime in excess of 300 runs.

Finally, another fluorescence IA is described by Evans et al.[52] using the antiepileptic drug phenytoin and POROS II A material as the solid phase. A mixture of analyte marker with rhodamine, analyte, and policlonal Abs (antyphenytoin) is passed through the column after a 2-min incubation time. Protein A links the conjugates Ab-Ag labeled, Ab-Ag, and Ab in excess. When a chaiotropic (citrate buffer/HCl pH 2.5) is applied, the retained species are eluted and a fluorescent signal is generated. The detection limit was 0.8 ng/ml of phenytoin, rsd (n = 10) was 3.2 to 6% and recoveries were 115 to 124%. The total assay time was 3.5 min and was applied to the analysis of serum. The columns have an active life of about 300 determinations.

The support shows good performances if compared with CPG, due to the combination of pores, which unlocks the interior of the particle, thus providing resolution and capacity unaffected by flow rate. This transport process in CPG is slow, especially for large molecules such as antibody-analyte complexes. The proposed format is very versatile and may be used with any analyte, provided that the antibodies are available due to the property of protein A of binding to the antibodies generally by the fragment Fc.

B. Drug Applications

Seven papers have been published in this field. All of them are centered on the determination of haptens, except one that analyzes the protein antigen hirudin; among them, there are drugs (cocaine) and pharmaceuticals (anticoagulants, anti-AIDS, anticancerigens, steroidal cardiac glycoside, and an antiepileptic). The main contribution of this work is developing flow formats, in most cases being the analyte used as model in artificial samples.

The contributions of Ogert et al.[53] and Rabbany et al.[54] propose a method for analysis of cocaine and its major metabollite, benzoylecgonine. The format, which is very interesting, immobilizes M_{Ab} derived from benzoylecgonine on sepharose. The antibody is saturated with fluorescein cadaverine benzoylecgonine so that the analyte displaces part of the conjugate and yields a positive sign of fluorescence. The resulting method is very good concerning sensitivity, comparable to that given by radioimmunoassay, with a linear range (0 to 200 ng/ml) and carryover lower than 0.7%. The life of the column, which does not need chaiotropics, is 55 determinations when operated under the most favorable conditions. Each determination needs less than 1 min, which is really astounding compared with the times required in FIIA.

A similar format was proposed by Gunaratna and Wilson[55] in an excellent work. They have prepare a CDI-activated Reacti-Gel HW 65F column on which the haptene (α-difluorometil) ornitine (DMFO) is immobilized. The policlonal antibody-enzyme conjugate is prepared, reducing the interchain disulfide bounds to produce at half-molecule, which is labeled with the enzyme HRP and this tracer is then mixed with the analyte. The unretained fraction of tracer in the column is revealed, then adding the enzymatic substrate. A fluorescent signal proportional to the concentration of analyte is then produced. The column has sufficient capacity to allow for 50 determinations, after which it is regenerated with 0.1 *M* phosphate buffer (pH 2.2).

Operating under these conditions, the column has a 1 month active life. The method is very sensitive (detection limit, 2.10^{-11} *M* of DFMO), with an analysis frequency of 9 to 10 h^{-1}. Both accuracy and precision are good, and the carryover of 2 to 4%. The DMFO values obtained from plasma samples with this method correlate well with the results obtained by the chromatographic method. A comparison of the proposals of Ogert et al.[53] and Gunaratna and Wilson[55] shows that both give rise to similar satisfactory performances, although the first one is simpler to develop, and the analysis frequency is much higher. The argument by Gutaratna and Wilson that justifies the use of monovalent antibodies to obtain 1:1 stoichiometry of antibody-antigen conjugate does not seem particularily satisfactory due to its greater complexity concerning the development of the method, especially taking into account the results of Ogert, which also agree with those of Freitag et al.[56]

For the determination of hirudin, Oroszlan et al.[57] set up an optosensor with fluorescent detection and a sandwich format. It uses a monoclonal antibody that covalently immobilizes onto the surface of the sensor, and the analyte is then incubated for 10 min with the M_{Ab}. Afterward, the second antibody marked with fluorescein is added, which generates a fluorescent signal on the surface of the sensor that is read for 5 min. The sensor is finally regenerated with glycine/HCl at pH 2.5. The sensitivity is very high and the dynamic range covers 0 to 60 ng/ml with a precision of approximately 5%. It has the drawback that the activity of the reactor drifts slow but constantly, allowing around 90 determinations. The sandwich format is advantageous in obtaining good sensitivities, but it cannot be applied to haptens, as these are small molecules and it is difficult for them to have more than one epitopique determinant and, consequently, antibodies for them.

Hunt et al.[58] also use two antibodies but not a sandwich format. They propose a method for azidothymidine (AZT) determination that, although very sensitive (d.l. 6.57 10^{-11} *M* of AZT) is scarcely practical due to the fact that the activity of the bead decreases rapidly (maximum four regenerations) and several reactors must be used, previously standardized, to calibrate and analyze a sample. Furthermore, the analysis frequency is very low (some 25 min per determination) and it is necessary to use washings and chaiotropics with scarcely satisfactory results. The format consists of immobilizing an anti-rabbit AZT antibody (obtained of goat antirabbit antibody) on CPG via avidin-biotin. This antibody (P_{Ab}) recognizes the anti-AZT rabbit antibodies. After removing the unconjugated antibody, peroxidase-labeled AZT is added. Afterward the analyte is added, which partially displaces AZT-HRP so that the analytical signal is finally measured electrochemically. The enzymatic activity was indicated by the relative increase in oxygen tension due to peroxidase-catalyzed decomposition of H_2O_2 to oxygen and water; the method was employed to determine AZT in Retrovir capsules. Finally, Kaneki et al.[59] set up a capillary enzyme immunoassay with flow injection analysis for digoxin using a sequential saturation technique. They prepared an open tubular reactor formed by a capillary (10 cm × 0.53 mm of internal diameter) onto which the policlonal antidigoxins Abs are immobilized. Then the analyte is passed followed by the tracer, so that the reactor becomes saturated. Once the excess of enzyme conjugate has been eliminated, it is revealed electrochemically. The sensitivity is extraordinary (d.l. 10 pg/ml), five times higher than that of the ELISA technique and the time used is 25 min per determination; moreover, the dynamical range covers three orders of magnitude (10 to 1000 pg/ml). The main problem of this format is that the increase surface area-to-volume ratio of the microcapillary reactor increases the sensitivity and undesirable nonspecific interactions between the enzyme conjugates and

components of the immunoreactor other than the antibody paratope. Polyethylene glycol was used as coating and to extend for the attachment of the capture antibody. The sensitivity is very much improved, mainly due to the large surface area-to-volume ratio of the capillary immunoreactor.

Despite this, the method seems scarcely practical owing to the lack of reproducibility in the results as a consequence of the different capillary immunoreactor tubes used for each measurement.

C. Environmental Applications: Pesticides

In this area, most of the published works have been developed[12,60-65] by the group headed by Schmid. The rest[66-68] correspond to Camman et al., Oroszlan et al.,[69] and Cámara et al.[70] All but the last one are applied to triazines either in artificial samples or in water. They are, as a rule, basic works with a certain degree of difficulty when applied in real sample analysis.

The work of Schmid begin[60] with the development of an optical sensor that operates with a competitive format using monoclonals. Special attention is paid to the study of the nature of the interaction of hapten-antibody. In the following work,[61] preliminary results are given on the automation through heterogeneous formats and the development of biosensors. In the first case a replaceable (after each trial) membrane reactor is developed. The results are comparable to those given by the ELISA formats.

The inmunosensor is a flowthrough system,[12] basically consisting of a fiber optic onto which a derivative from the atrazine is immobilized. The monoclonal antibody is labeled with FITC and mixed with the analyte that competes with the antigen for the antibody. The results are good with a detection limit of 1 μg/l, allowing 300 determinations without a loss of activity. The analysis times are very long and the regeneration of the optrode is performed successfully by incubation with proteinase for 10 min.

In a further work, Wittmann and Schmid[62] again use the membrane reactor on which they immobilize policlonal antibodies against atrazine via protein A. The hapten is labeled with peroxidase and the detection is carried out fluorimetrically. The results show a good sensitivity, 0.1 μg/l for C 50%, and a variation coefficient of 9%. However, it is recommended only for screening. Due to its mechanical complexity, this proposal has proven to be hardly useful and has not been employed again by this group of researchers, who propose the use of packed-bead reactors as more advantageous.

In 1994, Schmid and Bier[63] developed an inmunosensor for triazines using monoclonal Abs and covalently immobilizing the hapten on the sensitive part of a planar monomode waveguide. This is the grating in the transducer used. They shorten the analysis time to 15 min by making kinetic measures and increased the sensitivity, detecting concentrations of 15 n*M* of terbutrine. In this work, proteinase in solution is also used with good results to regenerate the reactor. The sensor shows interferences of prometryn, terbutylazine, and atrazine.

In another work, Bier et al.[64] focus on the regeneration and calibration of the sensor previously described. By testing different chaiotropics, proteinase is selected as most effective. They also succeed in reducing the detection limit (15 μg/l) and the analysis time (30 min). Operating under these conditions, the sensor can carry out 600 analysis cycles without a remarkable loss in activity.

The last paper of Schmid et al.[65] deals with the analysis of triazines in soils and waters through a competitive heterogeneous format, using monoclonal and policlonal antibodies immobilized on polyestirene and CPG, respectively, with fluorescent detection. Triazine labeled with peroxidase is

employed as tracer. The results obtained with P_{Abs} are the best ones, covering a wide dynamical range of 0.001 μg/l to 1 μg/l. The regeneration of the system is complete and very rapid (1.5 min) using glycine 0.01 *M*/ HCl, pH 2.

The group headed by Cammann is working[66-68] on the development of FI methods for triazines using an europium (III) complex (BCPDA) as a fluorescent marker. The analyte, atrazine, is made to react with albumine or biocytin bound to streptavidin labeled with BCPDA. The best results are obtained with this last coupling reaction.[66] In another work,[67] the authors compare the results obtained after marking the antigen with ferrocene and enzymes and the antibody with the Eu(III) complex. In another contribution,[68] a sensor is developed by immobilizing a derivative from triazine on oxiran acrylic. The format employs an affinity column containing the hapten, which is saturated with the antibody labeled with a Eu(III) chelate. The displacement of the labeled antibody caused by the analyte generates the analytical sign without the need for removing the bound species. Complete and rigorous investigation succeeds in setting up a reliable method with a detection limit of 1 μg atrazine per liter and a linear dynamic range of three orders of magnitude: 10 to 10^4 μg/l. The useful life of the column is 2 weeks. The analysis frequency is really low (75 min per determination).

Oroszlan et al.[69] prepare a sensor for atrazine. The system is based on an evanescent wave fiber optic and operates according to a competitive heterogeneous format with fluorescent detection. The monoclonal antibody is immobilized by physical adsorption on the fiber optic. The regeneration of the sensor is good when 0.1 *M* $MgCl_2$, pH 2.5 is used, although there is a linear loss of activity. The use of bovine serum albumine (BSA) in the buffer (100 μg/ml) had a significant influence on the lifetime of the sensor. The results obtained are satisfactory. Samples with 50 n*M* of atrazine being analyzed after 200 cycles, with a precision of 1.9% (C.V.). The method presents some advantages when compared with the ELISA formats due to its greater speed and considerable increase in precision.

Finally, Cámara et al.[70] analyze paraquat and parathion in water. They describe the synthesis of a fluorescent marker derived from the pesticides target with acriflavine Rhodamine B isothiocynate and Lucifer yellow as labels. The assay is based on an heterogeneous fluorescence competitive format. A bead-packed reactor with protein A immobilized on CPG is prepared, and the FI variables are studied as well as those related to the efficiency of the binding and elution between receptors and ligand.

D. Bioprocess Monitoring

In order to improve biotechnological production processes, enhanced on-line analysis techniques such as flow injection and better process control are necessary. On-line protein monitoring has been developed with turbidimetric immunoassays as well as with homogeneous and heterogeneous immnunoassays.[71] Despite numerous publications reporting automated IAs on model systems, the number of immunochemical systems applied to real samples, especially to bioprocess monitoring, is low. All of them are based on the principle of FI.[72-74]

Immunoanalysis for process control is reviewed by Mattiasson and Hakanson,[75] and the development of nonequilibrium IA from a manually operated FI system to a completely computerized system for sample analysis, as well as calibration and data evaluation, are also discussed.

Even if the area is young, much progress has been achieved and one can foresee an interesting future development in this area. The recent application of immunoanalysis for research and process monitoring in bio-

technology using FI are discussed. Emphasis is put on systems for on-line bioprocess monitoring, and examples are given for α-amylase, pullulanase, mouse IgG, antithrombin, etc.

Immunoanalytical techniques such as ELISA are often used for the detection of proteins produced in culture process. Owing to the difficulty of automating the traditional time-consuming ELISA, there is a growing demand for a suitable on-line monitoring method.

Flow-ELISA may be attractive for monitoring cultures as well as down-stream processes. In fermentations, it is interesting to record the appearance and concentration of certain substances. In the area of downstream processing, it is desirable to trace a target product through the different unit operations.

The work of Nilsson et al.[76] clearly demonstrates that flow-ELISA gives a powerful immunoanalytical technique that can be automated for bioprocess monitoring and control, allowing quick analysis of macromolecules in complex mixtures. The assay is based on the repeated use of a preparation of immobilized Abs. Stability in the regeneration step is a prerequisite and was achieved by intermittent recalibration of the system. In previous papers, the authors[77] have reported the development of flow-ELISA into a fully computerized analytical procedure capable of registering dynamic changes in concentration.

Another paper by Mattiasson and Hakanson[75] deals with optimization and characterization of this computerized system. A sample containing α-amilase passed through a column containing Ab immobilized on CNBr activated sepharose CL-4B. Afterward, α-amylase conjugated with peroxidase was added and bound by Abs not occupied by the sample. A pulse of substrate solution was injected and reacted with bound enzyme, giving a colored product detected spectrophotometrically, and the bound α-amylase was eluted from the column. A complete measuring cycle took 300 s. The range studied was 0 to 1 g α-amylase per liter. More than 250 samples were run through the column.

A new, simple sandwich assay with protein A immobilized on a solid support (CNBr-activated sepharose 4B) for the determination of various IgGs was established.[78] β-Galactosidase was used as enzyme marker and lactose as substrate. In a different column the generated glucose was analyzed via an enzyme thermisor device. This calorimetric immunoassay is useful for bioprocess monitoring when high Ab concentrations are obtained. Over 50 analysis cycles could be performed with this system (deviation range of 4 to 5%) with a standard for the rabbit IgG quantitation;[79] the times required for one assay cycle are 8 and 16 min at flow rates of 1.2 and 0.6 ml/min, respectively.

Middendorf et al.[80,81] investigated the thermostable proteins pullulanase, IgG, antithrombin III, and recombinant tissue-type plasminogen activator by means of heterogeneous and turbidimetric immuno-FI methods. In the cases of pullulanase and monoclonal mouse IgG, turbidimetric FIIA was used for on-line analysis of the culture process.

In the heterogeneous assay a mixture of reagent and analyte passes through a column with immobilized Ab in which a competitive reaction takes place. As the fluid leaves the column, the concentration of the FITC-labeled Ag is measured fluorometrically. The measuring range (μg/ml) is very suitable for bioprocess control, on-line measurement, and automatic control being possible as with any FI system. The column could be regenerated more than 80 times. After 80 cycles, 70 to 80% of the initial signal was retained.

In the turbidimetric FIIA (TIA) sample is mixed with the reagent Ag for Ab/Ag interaction and flows into the reaction coil. The stopped-flow mode is used to increase the spectrophotometric signal due to a longer incubation time. An advantage is the short

assay time between injection and detection (1 to 5 min), and the major drawback is the relatively high reagent cost. It is possible to measure in the range (μg/ml) in which protein concentrations occur during cultures and no dilution is necessary.

Two flow-injection immunoanalysis systems for the observation of the production of the mouse IgG during the culture of hybridoma cells in a perfusion reactor are presented by Gebbert et al.[82,83] One is based on the principle of an electrolytic capacitor. Antibody or antigen was immobilized onto the tantalum oxide surface, and the binding of the corresponding analyte resulted in a modification of the electrical capacitance proportional to the amount of bound mouse IgG. The principle and theoretical basis of the capacitance system were described previously.[84] The measuring range was 2 to 200 μg/ml and the relative error was 8 to 15%. After more than 130 cycles, the loss of activity was 20%. The effectiveness of binding with respect to the Ab load and regenerability of the sensor surface can be improved by designing immobilization methods, especially for the metal oxide surface, because Ab are immobilized according to the well-developed methods for silica surfaces.

The second FIIA system is based on the principle of a sandwich ELISA with spectrofluorimetric detection. Abs were immobilized on oxiran-acryclic beads filled in a column, and β–galactosidase labeled Abs were used as secondary Abs. The automation of the system was performed in the conventionally two-step mode. Using stopped flow, the total assay cycle was 11 min. The measuring range was 1 to 1000 μg/ml and the relative error was 5%. A 26% loss of activity was observed after more than 180 assay cycles.

Immunosensors used in FI systems offer interesting advantages over conventional analysis techniques for bioprocess monitoring. Biosensors have to be adapted to the bioprocess under real culture conditions to yield reliable results. Great expectations have been placed on immunosensors for on-line bioprocess monitoring. By combining a highly selective biological compound such as Ab with various types of transducers, biosensors provide the ability to analyze single analytes in the very complex fermentation medium in a highly selective manner.

A review about the FI biosensors for bioprocess monitoring in Germany and some applications of immunoanalysis systems for real process monitoring is presented by Scheper.[85]

Optical FI immunosensors are also suitable for biotechnological applications. A solid phase assay for biotin on silica fiber was developed by Schulze et al.[86] based on the difference in the dissociation constants of Extr-Avidin-Biotin and Extr-Avidin-Imminobiotin. The fiber is silanized and coated with BSA-Imminobiotin conjugates. In order to employ FITC as a label, a halogen lamp is used. The maximum binding capacity of tested fibers is around 40 pmol of Extr-Avidin-FITC conjugates. The lowest detection limit is 10 pmol of Extr-Avidin-FITC.

Recently, a grating coupler immunosensor was used[87] for the on-line determination of monoclonal Abs produced in a perfused animal cell bioreactor. The device was connected with the culture vessel via an FI system, which was controlled automatically. Specific antimouse IgG antibodies were immobilized on the surface of a sensor chip. After injection of the sample, the binding of mouse IgG was observed in real time. The regeneration on the binding sites of the immobilized Abs allowed the on-line detection of produced M_{Abs} in the range of 10 to 150 μg/ml. Unlike other techniques coupled to bioprocesses, the developed method represents a regenerable direct immunosensor.

Advanced control techniques are being developed and have been used in laboratories. However, they are not yet reliable enough for application in biotechnological

production processes. It is expected that their reliability will be improved within a few years and that they will then be introduced into industrial practice. On-line process analysis will be extended to additional medium components by using biosensors integrated into FI systems, and analysis instruments will be made more intelligent by means of suitable software.

E. Miscellaneous

An immunosensing system for the detection of HIV-specific antibodies on a combination of the ELISA principle and an ISFET flow injection set up is presented by Aberl et al.[88,89] A cartridge containing Ag molecules immobilized onto an activated glass surface was chosen because it shows several advantages with respect to assayed cellulose nitrate membranes. In this system, urease is used as a marker enzyme, which catalyzes the hydrolysis of urea yielding a pH-shift detectable with an ISFET sensor.

Peptide antigens from the HIV p24 and murine M_{Abs} were chosen as models. Compared with the microtiter plate ELISA, the ISFET-FI-ELISA shows a slightly lower sensitivity, but improvements could be obtained by using packed bed columns containing CPG or latex spheres as support material. The authors point out that future advantages could arise from an extensive miniaturization and automation of the whole ISFET-FI system, for example, by means of silicon micromachining.

A prototype of continuous flow immunosensor for detection of trinitrotoluene (TNT) and dinitrotoluene (DNT) is described by Whelan et al.[90] Sepharose coated with M_{Ab} and saturated with fluorescein is used. The signal generated in a competitive format is detected using a fluorometer and is proportional to the concentration of unlabeled Ag over a range of 10 to 40 ng/ml for TNT and between 20 and 1200 ng/ml for DNT. The sensitivity is increased when 12.5% ethanol is introduced into the buffer flow stream.

The flow immnunosensor provides the means to detect trace levels of explosives in times below 1 min. This flow immunosensor prototype was assembled with off-the-self components at a cost of less than $15,000 (U.S.).

Sadik and Wallace[91] have developed a rapid, sensitive, and reproducible detection method for human serum albumin (HSA) based on the use of polypyrrole HSA with pulsed amperometric detection in an FI mode. The antibody containing electrodes was prepared by galvanostatically electropolymerising a pyrrole monomer from an aqueous solution containing anti-HSA solution onto a platinum substrate. The interaction Ab-Ag is encouraged with more positive potential applied. A pulse potential hydrodynamic voltammogram was obtained and gives an adequate sensitivity and resolution for FI. The reproducibility obtained was ±5% in the range 5 to 50 ppm protein and detection limits of less than 1 mg/l were estimated.

An immunoenzymatic detector system has been developed by Gorovits et al.[92] using human IgG as a model antigen. Ab covalently photoimmobilized onto a membrane was placed into an ultranarrow flow cuvette and a solution of Ag and Ab-peroxidase conjugate was passed through the cell. After washing, the membrane was placed into the substrate solution and the developed color was recorded spectrophotometrically. The lowest detection limit was 5.10^{-11} *M* and the overall analysis time was 10 min.

The specially constructed cuvette consisted of two plates that could be bolted together tightly. There was a small flow channel with a depth of 0.5 mm between two spacers placed between the plates. The surface dimensions of the working space in the cuvette were aproximately $10 \times 2 \times 0.5$ mm.

Finally, Brecht et al.[93,94] have shown that spectral interferometry allows on-line moni-

toring of immunoreactions. The change in the thickness of the monitored interference layer due to protein mass deposition is used for quantitative evaluation by means of a diode array spectrophotometer. With this detection principle no labeling compounds are necessary. All investigations were based on a solid phase adsorption assay protocol. The major test system was rabbit-IgG, policlonal goat antirabbit-IgG. The immobilization of the antigen as reported is of limited interest for practical applications. However, it has the advantage of giving a high density of binding sites at the sensor surface. Two flow regimes (continuous and alternating) were applied with comparable results (detection limit is in the range of 0.1 μg/ml IgG). Sample consumption is 10 ml per test with continuous flow and less than 2 ml for reversed flow.

II. NEW TRENDS

Techniques for the modification or immobilization of Ab remain an area of active research. Applications of these methods include their use in the attachment of Ab to drugs and labels or solid-phase supports. The most common approach for the site-specific modification of Abs involves the oxidation of Ab carbohydrate residues with periodate or enzymatic systems. This forms aldehyde groups at relatively well-defined locations on the Ab, which then react with labels or supports containing active amine or hydrazide groups. In order to control and monitor this oxidation process, a method is required that can quickly and conveniently determine the number of aldehyde groups generated per Ab. Wolfe and Hage[95] describe an automated system for the determination of Ab oxidation based on FI. As in earlier methods, this approach will detect aldehyde groups by labeling Ab with Lucifer Yellow CH (LyCH), but the amount of Ab will now be detected by using a flowthrough bicinchoninic acid (BCA) protein assay. The rabbit IgG was oxidized with periodic acid and labeled with LyCH as described previously.[96] The analysis time was 2 min per 20 μl. The detection limits for rabbit immunoglobulin (IgG) and LyCH were 1.10^{-8} and 4.10^{-7} *M*, respectively. The dynamic ranges for IgG and LyCH extended to 2.10^{-5} and 7.10^{-3} *M*. The within-run precision was 5% or less for both analytes. Studies with known LyCH/Ab mixtures indicated that the FI system had greater accuracy than manual methods at high LyCH levels.

Although this work examined the use of the FI system with rabbit Abs, the same approach could easily be adapted for use in monitoring other types of oxidized Ab or glycoproteins.

An important parameter for the reliability of heterogeneous EIA is the quality of the solid-phase support for the primary Ab or Ag. Two basic choices may be considered to regenerate the solid phase at the end of the assay: either the reuse of the primary Ab already linked on the support or the immobilization of a new Ab after a complete cleaning of the support.

In the former instance, a washing step with chaiotropic ions or buffers at low pH and high ionic strength is used to break the Ab-Ag bond. This method, which is the most commonly employed, especially in the development of immunosensors, has been tested — apparently with success — with different kinds of solid phases, including polysaccharide gels, electrode or membrane interfaces, fiber-optic interfaces, etc.[97] However, some reviews in the affinity chromatography field indicate that successful regeneration, including the negligible irreversible denaturalization of the immobilized Abs, needs a relatively low level of affinity between Ag and Ab. This is a contradiction with the high-affinity constants required for good sensitivity of ELISA.

In the latter instance, the strategy is to completely eliminate the immunological

stacking by unhooking the primary Ab. A possible solution is to use a reversible covalent linkage such as a disulfide bond between the solid phase and this primary Ab and a dithiothreitol washing at the end of the assay. A more drastic method is to attack the solid phase surface itself.[98,99] An automatic apparatus has been developed around an electrochemical flow cell fed with samples and reagents according to the principles of FI. Using rabbit IgG as analyte and glucose oxidase as label, series of 150 successive assays were conducted on the same electrode before it was polished again.[97] A prototype automatic electrochemical IA analyzer was developed by SERES (France). The EC cell was a thin-layer flow cell made of PTFE with a dead volume of 21 µl. The working electrode was a glassy carbon disc 3 mm in diameter. Two separate lines alternatively fed the cell. Along the first one, an automatic valve, a sampler, and a peristaltic pump injected successively the primary Ab solution, the blocking buffer, the sample solution, the labeled Ab solution, and the rinsing buffer solution. On the second flow line, two peristaltic pumps and a double-channel valve allowed the successive injection of the benzoquinone and the glucose-benzoquinone solutions for the enzyme activity determination and the acidic solution used for the EC cleaning of the carbon electrode. The time for a complete cycle of measurement was 55 min.

The separation of the labeled bound reactant, Ab or Ag, and the labeled free reactant is a major disadvantage of heterogeneous IA. Recently, fully automated assays, which are based on high-performance immunoaffinity chromatography (HPIC) and capillary electrophoresis (CE), have been developed.[100]

Capillary electrophoresis has emerged as a powerful separation technique. Limited sample volumes can be analyzed as subnanoliter injection volumes are an inherent feature of the small (5 to 20 µm i.d.) capillaries.[101,102] Despite the fact that CE is compatible with many detection systems, its application to IAs has yet several problems such as the nonspecific adsorption in the inner surface of the capillary and the overheating of the samples. Laser-induced fluorescence coupled to CE was used to determine several analytes and major proteins at fmol and amol levels.[103,104] Schultz and Kennedy[100] have proposed a method for insulin using capillary zone electrophoresis with fluorescent detection and a homogeneous competitive format that allows the determination of a great number of antigens.

An alternative that does not require bound/free separation is the use of latex particle suspensions. Latex immunoassay is a general method based on agglutination by reaction with an Ag of calibrated latex particles coated with a specific Ab. The method can quantify a wide variety of protein antigens.[105] The assay is usually performed in a simple continuous flow system by incubating the reaction mixture in a heated mixing coil for about 30 min. The incubation time can be shortened by using an alternating field that enhances mixing and thus the agglutination of the latex particles.[45] The agglutination rate can be measured by nephelometry, turbidimetry, and by particle-counting instruments. For all methods the precision in a series of measurements and from day to day is in the range 5 to 10%. However, particle counting assays are three orders of magnitude more sensitive compared with nephelometry or tubidimetry. Detection limits that range from 10^{-10} to 10^{-12} *M* are commonly achieved.

Recently, Rosezweig and Yeung[106] reported a successful application of particle counting IA for the analysis of intracellular components at the zeptomole (10^{-21} mol) level. A 20-µm-i.d. capillary was used as the continuous flow system. Light scattering of the laser beam was used to monitor the agglutination of particles when the antigen is present. Glucose-6-phosphate dehydrogenase

(G6PDH) was chosen as the test compound. The method was applied to the determination of G6PDH in single red blood cells in a linear range between 10^{-21} and 10^{-19} mol and can be applied to the determination of any antigen, including non-protein compounds.

Many detection schemes and assays have been developed since the introduction of FIIA in 1980. Advances in FI also continue and techniques such as double injection, immunodetection (ID), and sequential injection (SI) offers new ways in immunoassay.

Immunodetection is a novel approach that combines the advantages of both LC and IA to perform a fully automated real-time measurements of analyte concentration. The ID technique is based on the use of recently developed, very high-speed LC materials. These perfusion chromatography media, when used in combination with Abs or other ligands, allow for a complete analyte capture in seconds. The ID-sensing device consists of three elements: the immobilized ligand, a flowthrough particle, and a flowthrough cartridge. The cartridge is intended for reuse hundreds of times, and long incubation steps are eliminated as residence times of less than 1 s are sufficient for complete capture. The ID assays are developed on HPLC instruments, which are in turn designed for automation.

Afeyan et al.[107] describe the principles of ID, its performance characteristics, aspects of automation, and novel applications. An ID assay was developed by these authors for the direct measurement of human serum albumin (HSA). A cartridge with covalently immobilized anti-HSA was used. A typical assay run took 1 min to complete and was sensitive down to 100 to 500 ng of analyte. Detection of HSA was performed after elution with 5 m*M* HCl by measuring UV absorbance. The same cartridge was used 200 consecutive times with HSA as the sample.

The sequential injection method uses immunomagnetic beads that do not require in-line regeneration by creating a renewable reaction surface that can be fluidically manipulated.[7] Recently, Pollema and Ruzicka[108] introduce a new methodology for carrying out heterogeneous immunoassays automatically, using the FI technique on a renewable surface. Flow injection renewable surface inmunoassay (FIRSI) uses a heterogeneous format that does not replace the support after each assay. The detection is made by direct measurement with a fluorescent microscope. With this system, a good sample throughput is achieved.

This study introduces the principle of FIRSI and explores the experimental variables affecting the system, such as flow rate, contact time, and the amount of bead suspension introduced into the detection region. Different assay protocols (competitive and sandwich assay) using fluorescent detection are also discussed.[109] The most important drawbacks of FIRSI are related with the need to keep a homogeneous bead suspended in order to maintain a constant activity of the beads and that of using sophisticated detectors having difficulties to obtain sufficiently reproducible signals on variable geometry layers.

Exciting topics for further research include the use of enzymatic labels. Analytes having low molecular mass must be coupled to carriers to stimulate Ab production. The selection of the appropriate target and the design and synthesis of the suitable hapten-protein conjugate are key steps in the development of new immunochemical methods. Strategies for designing and coupling haptens to carriers have been reviewed by van Emon et al.[110]

The effective distribution of Abs to high-demand analytes is essential if IAs are to become more widely used for routine analytical tasks.[111] Immunological techniques are strictly dependent on the availability of Abs at reasonable quality and price.

In practice, the acceptance of IA techniques requires its validation against well-established reference methods, usually chro-

matography, in complex matrices. In this sense, the recently published validations of IAs for analytes such as *S*-triazine herbicides should increase confidence in IAs methods to deal with real samples.[111]

IA methods are increasingly becoming important as screening techniques, especially when the number of samples to be analyzed is high and in environmental studies.

Future immunochemical techniques will be based on the use of recombinant antibodies, catalytic antibodies, multianalyte immunoassay, and metal complex detection. Although progress in biosensor development is expected to originate from contributions from both micromechanics and biotechnology, the latter is more innovative. At present, the following developments can be envisaged to revolutionize the performance of the biosensor. The main analytical parameters, that is, specifity, selectivity, and functional stability, will be improved by using chemically modified and genetically engineered immunoreagents. In addition to the new analytical potential offered by M_{Abs}, the modification of the Ab molecule will open up new horizons. This is true for bispecific Abs, which are a functional hybrid of enzymes and immunoglobulins, and the application of genetically engineered fragments, which could result in a decrease effect of nonspecific binding. Further progress is expected by applying tailor-made Abs.[112,113]

Research in the area of FI immunotechnology is currently focusing on a number of important topics relevant to Ab-based measurements and analysis. These include sensitivity, speed of analysis, signal-to-noise ratios, simplicity of use, and continuous monitoring capabilities. The application of IAs *in vivo* studies offers a special problem as a consequence of the difficulty of handling reagents in solution, calibration, regeneration, and so on. Solving these problems, for example, by reagent adsorption or microencapsulation, may widen the perspectives of using IA in such a complex field.

Faster and simpler immunoanalysis should be possible through the use of novel separation systems under study. In solid-phase immunoassays, repeated desorption and adsorption is the goal, but still the unsolved problems are still denaturization of the Ab protein structure by using appropriate chaiotropic solutions. A few reports have exploited Abs with relatively fast dissociation rates for efficient analytical applications. Successful reversible IA depends partly on screening Abs for those with a fast dissociation rate. In this sense, the available information for batch methods is not directly transferrable. The development of good flow formats often requires flashback in order to know the studied reactions in depth.

To summarize, FIIAs are a useful analytical tool that can complement conventional IA methods. Although the technique is not problem-free, none of its identified shortcomings appear to be insurmontable. Rapid results, ease of automation, inherently good sensitivity, and great specificity are a powerful combination delivered by FIIA.

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