

Immunoassays

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The term “immunoassay” refers to a diverse group of analytical techniques which are found throughout clinical laboratories. In this article, an immunoassay is defined as an analytical method that uses antibodies or antibody-related reagents for the determination of sample components. The selective nature of antibody binding allows these reagents to be employed in the development of methods that are highly specific and that can often be used directly with even complex biological matrixes such as blood, plasma, or urine. By combining the selectivity of antibody–analyte interactions with the vast array of antibodies that can be produced in nature and the availability of numerous readily detectable labels (e.g., radioisotopes or enzymes), immunoassays can be designed for a wide variety of analytes while also providing low limits of detection. These characteristics, along with the relatively low cost generally associated with these methods, have continued to make immunoassays a popular method in many clinical applications.

Many of the trends that were discussed in an earlier report on immunoassays (A1) have continued throughout the period of this current review. Some of these continuing trends include an emphasis on nonradioactive labels, more specific reagents, and improved formats for automating or performing immunoassays. The purpose of this article is to examine recent advances in the theory and analytical methodology of immunoassays, as represented by articles that appeared between January 1997 and December 1998. In this review, immunoassays are primarily categorized on the basis of the type of label that they employ (e.g., radioimmunoassays, enzyme immunoassays, etc.). Besides considering new developments that have occurred in each type of immunoassay, other topics will be discussed, such as advances that have been made in the theory of immunoassays or immunoassay reagents. Related items, including immunosensors and commercial immunoassay instrumentation, will be discussed elsewhere.

GENERAL BOOKS AND REVIEWS

A variety of materials appeared during this review period on the general topic of immunoassay methods. A book presenting an overview on immunoassays was edited by Price and Newman (A2). The present and possible future status of immunoassays was also discussed in several articles and book chapters (A3–A5). Appleby and Reischl reviewed some of the common types of immunoassays, with an emphasis on those that use monoclonal antibodies (A6). An overview of immunoassay automation was given by Gorman et al. (A7). General clinical applications of immunoassays that were reviewed included the use of these methods in toxicology and drug analysis (A8–A13) or in immunoscreening procedures (A14). Several papers focused on immunoassay measurements involving particular groups of clinical analytes, such as psychotropic drugs (A15), cardiac drugs (A16),

markers for myocardial damage (A17), gonadotropins (A18), prostanoids or leukotrienes (A19), and chiral drugs (A20). Related techniques that were discussed included those involving the detection of autoantibodies (A21), the monitoring of human exposure to environmental agents (A22), and the detection of cell surface molecules or the secreted cellular products (A23).

THEORY OF IMMUNOASSAYS

Many topics concerning the general use of immunoassays were examined in recent review articles. Examples include overviews that appeared on immunoassay design and optimization (A24), quality assurance (A25–A27) and quality control (A28), standardization (A29, A30), and data manipulation (A31). Quality management and standardization programs for protein immunoassays were described (A32), as was the standardization of immunoassays for prostate-specific antigen (A33) and steroids (A34). Witte et al. examined the frequency of occurrence of unacceptable results in the clinical use of immunoassays and other methods (A35). The use of the term “sensitivity” in immunoassays was discussed by Ekins and Edwards (A36). Sadler and co-workers gave an approach for estimating the total analytical error of immunoassays (A37), while Zweig and Kroll described a linear regression method for estimating the minimal detectable concentration for some hormone immunoassays (A38).

In addition, a number of advances in immunoassay theory were made. For instance, Jones et al. considered the use of pattern recognition and mixture analysis as a means for dealing with cross-reacting analytes in immunoassays (A39); similarly, Wittmann et al. described the use of a neural network for immunoassay pattern recognition (A40). Kinetic models were provided for a homogeneous two-site immunometric assay (A41) and a nephelometric immunoassay (A42). The role of thermally induced mass transport during immunoassay incubation was examined by Beumer and Timmerman (A43). Other papers discussed the relationship between antibody affinity and detection limits for solid-phase enzyme immunoassays (A44) and the effects of antibody–antigen kinetics or thermodynamics on the response of ELISA methods (A45) and radioimmunoassays (A46).

Various fundamental aspects of antibody–antigen reactions were discussed, as illustrated by a recent book chapter written by Van Regenmortel (A47). Reviews on the characterization of antibody–antigen interactions were provided by Mellado et al. (A48) and Roder and Markey (A49). Van Oss explained the role played by hydrophobic and hydrophilic forces in such reactions (A50), while Van Regenmortel discussed thermodynamic parameters that are important in immunoassays (A51). Pathak et al. discussed techniques for determining antibody affinity and affinity distributions (A52). A method was reported for the determination of affinity distributions that required only nanogram quantities of

material (A53), and antibody affinity analysis by ELISA was described (A54). The temperature dependence and/or thermodynamic properties for a number of specific systems were considered, including the reaction of antibodies with a membrane protein of *Neisseria meningitidis* (A55), theophylline (A56), egg white lysozyme (A57, A58), actin, myosin, and tubulin (A59), and duplex DNA (A60). Proba and co-workers looked at the changes in stability and folding that occurred in an antibody that lacked an essential cysteine in its VH region (A61). Wang et al. considered the effects of denaturants on antigen binding regions (A62). NMR was used to follow the change in amide hydrogen-exchange rates upon the binding of a Fv antibody fragment to lysozyme (A63). Atomic force microscopy was employed in studies of antigen binding forces to individual Fv fragments (A64) or to intact antibodies (A65, A66). Capillary electrophoresis was used to measure the binding affinities of several model antibody systems (A67–A69) and to follow pH-related isoform transitions in a monoclonal antibody (A70). Size-exclusion chromatography was utilized as a means for analyzing the masses of antibody–protein complexes (A71) and in determining antibody affinities (A72). Fourier transform infrared spectroscopy was used to analyze the secondary structure of humanized recombinant antibodies (A73). Electrostatic interactions were modeled for anti-nucleosome antibody Fv fragments (A74), and the folding kinetics of a Fv fragment were examined (A75). The use of multipin peptides for epitope mapping was also discussed (A76).

The kinetics of antibody–antigen reactions were discussed in many reports and in reviews by Karlsson and Roos (A77) and Van Oss (A78). The effects of high-pressure on antibody–antigen binding rates were examined by Green et al. (A79). A kinetic model for the binding of antibodies to multivalent antigens was presented (A80), and fractal analysis was used to describe the kinetic behavior of antibody–antigen systems on solid phases or biosensor surfaces (A81–A85). Numerous studies used surface plasmon resonance to examine antibody–antigen association and/or dissociation rates (A86–A94); this subject was also reviewed by Geddes and Lawrence (A95). In related work, surface plasmon resonance was combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the detection of antibody–antigen reactions on the surface of a fiber-optic probe (A96). Other methods that were used to investigate antibody–antigen kinetics included ELISA (A97), total internal reflection fluorescence microscopy (A98), a resonant mirror biosensor (A99), a flow injection chemiluminescent sensor (A100), and high-performance liquid chromatography based on immunoaffinity columns (A101).

The behavior of antibodies or antigens at surfaces is another area of continuing research. The characteristics of antibodies or proteins that are bound to microplates was discussed by Agnellini et al. (A102). Norde reviewed information related to the adsorption of antibodies or other proteins to polymers and colloids (A103). Scanning angle reflectometry was used to examine antibody–antigen layers on silica (A104). Atomic force microscopy was employed as a means for studying the packing of IgG antibodies and other model proteins on silicon surfaces (A105). Surface plasmon resonance (A106) and capacitive measurements (A107) were used to follow the behavior of antibodies on surfaces containing self-assembled monolayers. Three reports considered

the changes that occur in the binding properties of antibodies and/or antigens when they are adsorbed or immobilized to solid surfaces (A108–A110). The role played by the coupling method in determining binding capacity and ligand leakage was examined for immunoaffinity supports (A111), and studies were conducted on antibody–antigen reactions that take place in sol–gel matrixes (A112).

Possible interferences in immunoassays were discussed in many papers. This included the effects of endogenous agents such as thyroid hormone autoantibodies in thyroid-related immunoassays (A113), human anti-mouse antibodies (HAMA) and other heterophilic antibodies in assays that use reagent antibodies from mice or other nonhuman sources (A114–A118), anti-insulin antibodies in insulin immunometric assays (A119), sugars in methods that employ biotin–avidin or biotin–streptavidin as coupling agents (A120), and variability in plasma composition during the preparation of samples and standards for drug immunoassays (A121). Examples of exogenous agents that were considered as possible interferences included hemoglobin- and perfluorocarbon-based oxygen carriers (A122), buflomedil (in assays for tricyclic antidepressants) (A123), and chemicals or household agents that are commonly used as adulterants in drug immunoassays (A124).

ANTIBODIES, IMMUNOASSAY SUPPORTS, AND RELATED REAGENTS

The reagents that are used within an immunoassay make up a key factor in determining the ultimate sensitivity, selectivity, and limits of detection for the method. The antibody itself is the most important of these components, making it essential to consider in the assay's design. A series of books by Pound (A125), Delves (A126) and Lefkovits (A127) discussed various techniques in immunology and in the use or development of antibodies. Reviews also appeared on methods for generating multivalent or bispecific antibody fragments (A128), the production of human monoclonal antibodies from B-cells (A129) or transgenic mice (A130), antibody engineering (A131), and the use of anti-metatype antibodies in immunoassays (A132). Several immunoassay methods were developed that employed bispecific antibodies, including techniques reported for prostate-specific antigen (A133), β -microseminoprotein (A134), HIV gp120 and carcinoembryonic antigen (A135), thyroid-stimulating hormone (A136, A137), lactoferrin (A138), and α -endorphin (A139). In a similar fashion, genetically engineered single-chain antibody fragments were considered for use in immunoassays (A140–A144). The production and screening of phage display libraries for antibodies and antibody fragments was discussed in many articles (A145–A147), as was the production and analysis of antibodies generated by hybridoma cell cultures (A148–A152). The use of capillary electrophoresis for the characterization and analysis of antibodies was also reviewed (A153–A155) and employed in thermal stability studies of antibodies (A156).

Supports and coupling methods for immunoassays are two other factors to consider in immunoassay design. Work in this area included studies on the immobilization of antibodies or antigens to modified polystyrene or latex (A157–A162), polyacrylamide (A163, A164), acrolein copolymers (A165), poly(vinyl alcohol)–poly(acrylic acid) graft polymers (A166), nitrocellulose (A167), capillary wicks (A168), starburst dendrimers (A169), and

aluminum hydroxide (A170). Other studies considered coupling methods based on photoactivatable silanes or surface patterning (A171–A173) and on thiol silanes plus heterobifunctional cross-linkers (A174, A175). The use of magnetic particles as immunoassay supports was reviewed (A176, A177). Kinetic studies were performed on the oxidation of antibodies for coupling to hydrazide-activated materials (A178) and on the rate of covalent immobilization of these antibodies to small-diameter supports (A179). Related reports reviewed or examined the use of supports containing molecular imprints as antibody mimics in immunoassays (A180–A182).

The proper preparation and selection of antigens or conjugates is another item that can affect immunoassay performance. A review by Warnes et al. described the use of recombinant antigens as reagents in ELISA techniques (A183). The synthesis of mono- and bifunctional peptide–dextran conjugates was described for the immobilization of peptide antigens (A184), as was the in situ immobilization of antigens for organic-phase immunosupports (A185). Microbial transglutaminase was reported as a tool for the preparation of hapten–protein conjugates for immunoassays (A186). Ligand-modified phospholipids were employed for selective antibody precipitation (A187). The effects of long-term storage on immunoblots were studied (A188), and dot blots were used as a way of determining the extent of antibody/protein modification by amine-based coupling methods (A189).

RADIOIMMUNOASSAYS

Even though nonisotopic immunoassays are now used in the majority of clinical applications, methods based on radiolabels continue to play an important role in experimental and routine testing. The main techniques included in this group are the competitive binding radioimmunoassay (RIA), the immunoradiometric assay (IRMA), and the scintillation proximity assay (SPA). The radioimmunoassay (A190, A191) and scintillation proximity assay (A192) were both subjects of recent reviews. A paper on FlashPlate technology examined the use of specially prepared microplates that eliminate the need for scintillation additives to improve the detection of radiolabels in radioimmunoassays (A193). An improved count mode for the correction of color quenching in SPAs was also described (A194). A report appeared on the characterization of SPA membranes there were prepared from polysulfone polymer solutions (A195), and possible interference effects were discussed regarding the administration of contrast agents to patients who were later tested for various tumor markers by using immunoradiometric assays (A196). In addition, the effects of organic solvents on the calibration range of a radioimmunoassay were examined (A197).

ENZYME IMMUNOASSAYS

The majority of routine immunoassays consist of those that use enzymes as labels. The more common techniques in this group are the enzyme-linked immunosorbent assay (ELISA), the enzyme-monitored immunotest (EMIT), the competitive binding enzyme immunoassay (EIA), and the immunoenzymometric assay (IEMA). The general topic of enzyme immunoassay was the subject of a recent book chapter by Gosling (A198). The more specific subject of ELISA was reviewed by Kemeny (A199) and

Pathak et al. (A200), while Self et al. discussed enzyme systems that are used for signal amplification in ELISA techniques (A201).

There continue to be advances made in the substrates and enzymatic systems that are used for detection in enzyme immunoassays. For example, several new or improved substrates were reported for peroxidase (A202), β -D-galactosidase (A203), and alkaline phosphatase (A204, A205). An expression immunoassay was reported by White and Christopoulos that used DNA labels to generate multiple copies of the α -peptide of β -galactosidase for enhanced signal amplification (A206). An improved approach for the detection of peroxidase in immunoblots was described (A207), and the performance of affinity-purified glucose oxidase conjugates was evaluated in enzyme immunoassays (A208). Hemin was employed as an enzyme-mimic label in studies by Zhu et al. (A209). In addition, a method known as “timed-ELISA” was reported, in which the conversion of iodide to iodine in the presence of hydrogen peroxide was used as an internal clock in an enzyme immunoassay that employed catalase as the label (A210).

A number of alternative enzyme immunoassays remain of interest. One of these is the immune complex transfer enzyme immunoassay, which was used in several papers for the determination of HIV-1 p24 antigen and IgG antibodies to HIV-1 p17, p24, or reverse transcriptase (A211–A216). The combination of the polymerase chain reaction with ELISA detection (PCR-ELISA) also continued to grow in popularity, as indicated by papers in which this technique was adapted for the detection of amebiasis (A217), meningococcal infection (A218, A219), *Pneumocystis* (A220), *Coxiella burnetii* (A221), *Streptococcus pyogenes* (A222), plum pox potyvirus (A223), mycobacteria (A224), HIV-1 RNA (A225), and the telomerase activity of HeLa cells (A226), as well as for the genotyping of human VH3 genes (A227) and in the general quantitation of mRNA levels (A228). Other techniques such as “cell”- or “cellular”-ELISA (A229, A230), the enzyme-linked immunospot assay (ELISPOT) (A231), and enzyme-linked immunofilter assay (ELIFA) (A232) saw use in the detection of cells or cellular products.

Many reports during the last review period focused on alternative detection schemes or formats for enzyme immunoassays. For example, Dou and co-workers considered the use of surface-enhanced Raman scattering as a means for detecting azoaniline as a product generated by an enzyme immunoassay label (A233). A hand-held ion mobility mass spectrometer was similarly used by Smith et al. to detect phenol as the product of an ELISA method (A234). Immunoaffinity plates (A235), immunoaffinity chromatography (A236), and boronate affinity chromatography (A237) were all combined with enzyme immunoassays to improve analyte specificity. The development of various fluid elements was described for the automation of ELISA assays (A238) and a simple cloth-based enzyme immunoassay was reported for use in semiquantitative field testing (A239). Dual-enzyme labels were used with chromogenic substrates for the simultaneous detection of hepatitis B and C (A240). And finally, a novel conversion assay for homocysteine was reported in which the homocysteine was first converted enzymatically to S-adenosyl-L-homocysteine, followed by detection of this product by ELISA (A241).

FLUORESCENCE IMMUNOASSAYS

The group of techniques known as “fluorescence immunoassays” all have the common feature of employing a fluorescent signal for analyte detection. The common methods that make up this category of immunoassays include the competitive binding fluoroimmunoassay (FIA), the immunofluorometric assay (IFMA), the fluorescence polarization immunoassay (FPIA), and the time-resolved fluoroimmunoassay (TRFIA). Other related formats include the fluorescence excitation-transfer immunoassay (FETI), fluorescence modulation immunoassay (FMIA) and release fluoroimmunoassay (RFIA). Reviews dealing with some or all of these techniques were recently written by Wood and Barnard (A242) and Hemmila (A243).

Several new developments occurred in the area of fluorescence polarization immunoassays. Terpetschnig et al. reviewed clinical and biophysical applications of long-lifetime metal–ligand complexes, including their use for detection in FPIA (A244). New complexes that employed Re(I) or Ru(II) were described for the detection of high-molecular-weight analytes by FPIA (A245, A246). The synthesis of reagents for the detection of lead by FPIA was described (A247), as was the use of fluorescence polarization to characterize antibodies for immunoassay development (A248). And Seethala and Menzel developed an FPIA technique for measuring enzyme activity based on the generation of a product that competed with a labeled tracer for antibody binding in an immunoassay (A249).

Various reports also appeared on advances in the development and theory of time-resolved fluoroimmunoassays. Zuber and co-workers presented a mathematical model for describing the kinetics of a homogeneous immunometric assay with time-resolved fluorescence detection (A250). The use of lanthanide chelates as labels in TRFIA was discussed (A251). Another report examined the effects of coupling method on the behavior of europium chelates attached to antibodies (A252). Several papers described TRFIA assays that employed a new europium–BHHCT chelate as the label (A253–A255). Dual-label TRFIA methods were used for the simultaneous detection of phenytoin/phenobarbital (A256) and pregnancy-associated plasma protein A/free β -subunit human chorionic gonadotropin (A257). Finally, europium chelates and time-resolved microfluorometry were used in a noncompetitive immunoassay to detect the presence of prostate-specific antigen on individual microparticles (A258).

Work in the area of fluorescence immunoassay labels included the development of fluorescent tags based on glycoconjugated cyanine dyes (A259) and fluorinated fluoresceins (A260). The binding of fluorescein to anti-fluorescein antibodies was examined in the presence of reverse micelles (A261) and such micelles were used in immunoassays for various environmental agents (A262, A263). Two reviews discussed near-infrared fluorescent dyes as labels in immunoassays (A264) and in tumor detection or visualization (A265). Heptamethine cyanine dyes were one group of near-infrared fluorescent labels that were specifically examined for use in immunoassays (A266). Wright et al. described the use of rare earth elements embedded in crystalline particles as immunoassay labels that have multiphoton absorption of infrared light followed by phosphorescence emission in the visible range (A267).

There were various papers that examined new or improved applications of fluorescence immunoassays. For instance, Schuetz et al. compared the use of tetramethylrhodamine and phycoerythrin as labels in fluorescence microscopy for single-molecule detection (A268). Sauer and co-workers described the use of a pulsed semiconductor laser and fluorescence detection to monitor individual antibody molecules in serum (A269). Loeschner et al. reported the detection of single protein molecules at interfaces by employing antibody–analyte binding and fluorescence detection (A270, A271). Surface-enhanced fluorescence was used to detect labeled antibodies on metal nanoparticles (A272). A stopped-flow fluoroimmunoassay was developed for the detection of coproporphyrin in urine (A273), and enzyme-amplified lanthanide luminescence was used for signal generation in an immunoassay for interleukin-6 (A274). In work by Zhu et al., poly-(*N*-isopropylacrylamide) was employed as a thermal phase separating polymer and hemin was used as an enzyme-mimic label in the development of a fluorescence immunoassay for α -fetoprotein (A275). Hemin was also used as an enzyme mimic in a more standard solid-phase fluorescence immunoassay for hepatitis B surface antigen (A209).

CHEMILUMINESCENCE IMMUNOASSAYS

Techniques based on chemiluminescent and bioluminescent labels represent another growing group of nonisotopic immunoassays. The most common methods in this category are the competitive binding chemiluminescence immunoassay (CIA), the immunochemiluminometric assay (ICMA), and electrochemiluminescence immunoassays. The general subject of chemiluminescence immunoassays was reviewed in a book chapter by Weeks (A276) and in an article by Messeri (A277).

Several developments occurred in the field of enzyme-based bioluminescence immunoassays during the period of this review. A fusion protein containing biotin acceptor peptides and firefly luciferase was reported for the improved preparation of luciferase conjugates in immunoassays (A278). In addition, a number of new substrates for enzyme immunoassay labels, such as β -D-galactosidase (A203), and alkaline phosphatase (A204, A205), were designed for detection by chemiluminescence. A dual enzymatic system based on acetate kinase and a mutant thermostable firefly luciferase was employed in enzyme immunoassays for human growth hormone and human chorionic gonadotropin (A279). Some initial work was reported in the creation of catalytic antibodies that could generate chemiluminescence in the presence of 1,2-dioxetanes (A280). And a phenoxy-substituted acridinium ester was employed as an end point indicator in an enzyme immunoassay that used an alkaline phosphatase/glucose oxidase cascade system for detection (A281).

A number of additional papers examined the improvement or modification of other chemiluminescent labels. Reports appeared on the general use of electrochemiluminescence immunoassays (A282) and on specific techniques for the measurement of bacteria (A283) or recombinant proteins (A284). The electrochemically generated chemiluminescence of methyl-9-(*p*-formylphenyl)acridinium carboxylate fluorosulfonate was explored as a means for producing signals in immunoassays (A285). An octapeptide–aequorin fusion protein was developed for use as a labeled conjugate in a heterogeneous bioluminescence immunoassay

(A286), and *N*-(β -carboxypropionyl)luminol was considered as a label for chemiluminescence immunoassays (A287).

Various improvements occurred in experimental procedures for the detection of chemiluminescence. A flow injection method was described for the chemiluminescent detection of alkaline phosphatase tags in enzyme immunoassays (A288). Similarly, a postcolumn detection scheme for acridinium ester was combined with high-performance immunoaffinity chromatography in the development of a competitive binding immunoassay (A289) and one-site immunometric assay (A290) for thyroxine. The modification of a chemiluminescence ELISA method was described for measurement with a standard microplate reader (A291). Another paper examined the imaging of single cells by using an ultrasensitive chemiluminescent enzyme immunoassay and a photon-counting camera (A292). A new scanning device was described for detecting chemiluminescence on blotting membranes (A293). Finally, a review appeared on commercially available luminometers and imaging devices for bioluminescence/chemiluminescence assays (A294).

NONLABELED IMMUNOASSAYS

Nonlabeled immunoassay methods based on nephelometry, turbidimetry, particle counting, particle-enhanced light scattering, and latex agglutination are common in clinical laboratories. Other techniques that can be included in this category are immunoprecipitation and immunodiffusion. The overall topic of light-scattering immunoassays was recently discussed by Price and Newman (A295). Procedures for immunoprecipitation (A296) and coimmunoprecipitation (A297) were also described in recent papers. Quesada et al. presented a kinetic model for antibody–antigen reactions in particle-enhanced light-scattering immunoassays (A298), while Thompson et al. considered the mechanism and kinetics of an immunoinhibition particle-enhanced immunoassay (A299). The effects of hydration forces in latex agglutination immunoassays were examined by Molina-Bolivar and co-workers (A300). An improved data analysis method was reported for laser light-scattering immunoassays (A301). Light scattering from latex–latex or gold–latex dimers was studied in immunoassays and related methods (A302), while the effects of poly(vinyl alcohol) as a steric stabilizer for polypyrrole latex were examined in an immunochromatographic assay (A303). The simultaneous determination of methamphetamine, cocaine, and morphine in urine was accomplished by using colored particles in a latex agglutination immunoassay (A304). A method known as a liposome turbidometric assay (LTA) was reported that used antibody-coated liposomes to produce a change in light scattering as a result of antibody–analyte binding (A305). In addition, a new approach called a latex piezoelectric immunoassay (LPEIA) was described that uses a piezoelectric crystal for detection but does not require the immobilization of either antibody or analyte (A306).

ELECTROCHEMICAL IMMUNOASSAYS

Electrochemical immunoassays are a group of immunoanalytical techniques that have been the subject of continued research and development in recent years. These methods generally involve the production or use of an electrochemically active substance for signal generation. The advantages of this approach include the speed, accuracy, and precision with which many electrochemical measurements can be made. The general topic of electro-

chemical immunoassays was reviewed in a book chapter by Treloar et al. (A307). A review article by Cousino et al. discussed recent advances in this method, with an emphasis on its modification for the detection of analytes at the zeptomole level (A308). One approach for such assays is to use an enzyme label that generates an electroactive product; examples include methods that were reported for the determination of prostate-specific antigen (A309), *Helicobacter pylori*-specific IgG antibodies (A310), and luteinizing hormone (A311). An electrochemical enzyme immunoassay was also developed for use in a nonseparation, competitive binding format for small-molecule detection (A312). A related method, which employed hemoglobin as a catalyst for the creation of 2,3-diaminophenazine as an electroactive product, was described in a polarographic immunoassay for α -fetoprotein (A313). Other electrochemical immunoassays used copper ions as catalytic labels for the conversion of *o*-phenylenediamine to 2,3-diaminophenazine (A314, A315), iodinated erthyrosin B as a catalytic label for the As(III)–Ce(IV) redox system (A316), and 3,3',5,5'-tetramethylbenzidine as an electrochemical substrate for horseradish peroxidase (A317). Related developments in the electrochemical generation of light for immunoassay detection were discussed in the previous section on chemiluminescence immunoassays (A282–A285).

LIPOSOME IMMUNOASSAYS

These techniques are another group of alternative immunoassay methods that continue to be an active area of research. In such methods, liposomes are usually used to encapsulate a large amount of a traditional immunoassay label, such as a fluorescent compound or enzyme substrate, that is later released as a result of antigen–antibody binding at the liposome's surface. The general topic of liposome-based immunomigration assays was discussed by Roberts and Durst (A318). A fluorescence liposome immunoassay was reported in which carboxyfluorescein was used as an encapsulated marker for the detection of 2-phenyloxazalone (A319), and liposomes containing a europium chelate were used along with allophycocyanin to produce a signal in a fluorescence energy-transfer immunoassay for biotin (A320). Calcein was used as a fluorescent marker in a homogeneous immunoassay for insulin, which also employed phospholipase C to release this calcein from the liposomes (A321). Liposomes containing glucose 6-phosphate were used as labels in a glucose-6-phosphate dehydrogenase enzyme immunoassay for cytochrome *c* (A322). TEMPO choline was placed into liposomes as a marker and detected in an immunoassay by using a TEMPO choline-selective electrode (A323). In addition, a method for the analysis of C-reactive protein was developed in which a signal was produced by the change in turbidity that occurred upon the binding of the analyte to antibody-coated liposomes (A305).

CHROMATOGRAPHIC IMMUNOASSAYS

Another group of nontraditional immunoassays consists of those that are based on a flow-through system, such as liquid chromatography or flow injection analysis. Potential advantages of these flow-based immunoassays include their speed, precision, ease of automation, and ability to be coupled to other analytical methods. Techniques in this category include immunoaffinity chromatography (IAC), high-performance immunoaffinity chromatography (HPIAC), flow injection immunoanalysis (FIIA), flow

immunoassays, immunodetection, and immunoextraction. Hage reviewed analytical applications of these methods (A324), as well as their use in clinical or pharmaceutical testing (A290). Some other general reviews were written by Shahdeo and Karnes (A325) and Fintschenko and Wilson (A326). The more specific topic of immunodetection in HPLC and fluorescence immunoassay was reviewed by Krull et al. (A327). Gonzalez-Martinez and coauthors discussed methods for the desorption of retained analytes and the reuse of immunosorbents in flow-based systems (A328).

The direct detection of analytes as they are desorbed from immunoaffinity columns is the simplest approach that can be used in chromatographic immunoassays. This was utilized in a method reported for the determination of urinary leukotriene (A329) and in an assay for total and bioactive interleukin-2, where the bioactive fraction was measured by an on-line immobilized receptor cartridge (A330). In a related study, Phillips and Krum used a series of immunoaffinity columns and precolumn derivatization for the laser-induced fluorescence detection of 10 separate cytokines in a single 25- μ L sample (A331).

A second way in which immunoaffinity columns can be employed is to use them for off-line sample cleanup prior to analysis by a second technique. Off-line immunoextraction was included in new analytical techniques created for 19-nortestosterone and trenbolone (A332), dexamethasone (A333, A334), bufuralol and its metabolites (A335), vitamin D-related compounds (A336, A337), aflatoxicol (A338), agent S-8921 (A339), clenbuterol (A340), and ivermectin (A341), in addition to various synthetic corticosteroids (A342), anabolic steroids (A343), and growth-promoting drugs (A344). The samples in these reports were later analyzed by high-performance liquid chromatography (A332, A333, A335, A340), gas chromatography (A342), radioimmunoassay (A337, A339), or radioreceptor assays (A336). On-line immunoextraction was also used in several methods. These included immunoextraction/HPLC procedures developed for flunitrazepam and its metabolites (A345) and insulin in serum or in secretions from single islets of Langerhans (A346); immunoextraction/HPLC/mass spectrometric methods for β -agonists (A347), benzodiazepines (A348), corticosteroids (A349), or clenbuterol (A350); and immunoextraction/MALDI-mass spectrometric assays for the peptide SNX-111 (A351) or complement C1q (A352). The combination of immunoextraction and capillary electrophoresis was the subject of several additional reviews (A353, A354).

Low-concentration analytes can be determined in chromatographic immunoassays by means of either competitive binding or sandwich assay formats. A competitive binding immunoassay with an enzyme label was described for cortisol (A355), and chemiluminescence detection was employed in a competitive binding method for thyroxine (A289). Sandwich and noncompetitive enzyme immunoassays in flow-through systems were reported for ferritin (A356), IgG (A357), and digoxigenin (A358), while a one-site immunometric method with chemiluminescence detection was used in an assay for thyroxine (A290). The use of restricted-access columns for the separation of bound and free label in a flow immunoassay was demonstrated (A359). Fluorescein, peroxidase, and alkaline phosphatase were compared as labels in a noncompetitive flow-based immunoassay (A360); a similar comparison was made between carboxyfluorescein tags that were either used directly as labels or encapsulated as markers within

liposomes (A361). Perez and co-workers examined the use of immunomagnetic beads and electrochemical detection in a flow-based assay for viable *Escherichia coli* (A362), while Willumsen et al. designed a flow immunoassay with the complete renewal of the solid-phase prior to each sample injection (A363). In work by Hacker et al., a flow-based injection immunoassay was created that used the precipitation of antibody-analyte complexes as the means for analyte detection (A364).

ELECTROPHORETIC IMMUNOASSAYS

Capillary electrophoresis (CE) is another separation method that has been examined by numerous researchers for use in immunoassays. Attractive features of this approach include its potential separation speed, small sample size requirements, and need for small amounts of reagent. The topic of CE-based immunoassays was discussed in reviews by Schmalzing and Nashabeh (A365), Evangelista and Chen (A366), Bao (A367), Shahdeo and Karnes (A325), and Schultz et al. (A368). This subject was also included in a paper presenting an overview of clinical applications of microchip CE (A369).

The separation of free and bound analyte or label in a competitive binding method is one way in which CE can be used in immunoassays. This approach was utilized in analytical procedures developed for detecting thyroxine (A370), insulin secretion from islets of Langerhans (A371), methadone (A372), and scrapie-related proteins (A373). Most of these techniques used laser-induced fluorescence detection and fluorescein as the fluorescent tag, but other compounds can also be employed. One example is green fluorescent protein, which was studied by Korf and co-workers for possible use in CE immunoassays (A374).

A number of further developments occurred in this field during the last review period. For instance, microchips were used in the development of competitive binding CE immunoassays for thyroxine (A370) and theophylline (A375, A376). Weak monoclonal antibodies were immobilized within CE gels for the separation of carbohydrate antigens (A377), and immunosubtraction was combined with capillary electrophoresis for the clinical analysis of paraproteins (A378). The topic of immunoaffinity extraction combined with CE was reviewed (A353, A354), and a microdialysis-immunoaffinity method was combined with CE in order to examine neuropeptide-induced lymphocyte secretions (A379).

MISCELLANEOUS IMMUNOASSAYS

In addition to those techniques that have already been discussed, a large number of other immunoassay formats and designs have been studied. Multianalyte detection using microarrays was examined in two reviews (A380, A381). Disposable immunoassay devices (A382), microfabricated immunoassay systems (A383), and thin-film immunoassays (A384) were the subjects of additional reviews. The combined use of immunoaffinity capture with detection by the polymerase chain reaction (immuno-PCR) was reported in numerous articles (A385–A396). An immunoassay film badge was described for drug detection during breath analysis (A397); in similar work, a filter-based immunoassay was used to monitor air-borne drugs in the workplace (A398).

Other developments included the use of sub- and supercritical fluid extraction for sample pretreatment prior to immunoassays

(A399, A400). An immunoassay that used a water-immiscible solvent was investigated for use with hydrophobic haptens (A401). A sulfated antibody was employed to allow the separation of bound and free label by anion-exchange chromatography (A402). Several microsphere-based flow cytometry immunoassays were reported (A403, A404), and an upconverting near-infrared/visible phosphor reporter was described for use in such methods (A405). An immunoassay based on surface-enhanced infrared absorption spectroscopy was reported (A406), and surface-enhanced Raman microspectroscopy was combined with an immunoassay for determining membrane-bound enzymes in cells (A407). The remnant magnetization and magnetic relaxation of nanoparticles were examined as means for detection in immunoassays (A408), as was the infrared absorption of metal-carbonyl labels (A409). A method known as optical chromatography was described for the separation of bound and free beads as immunoassay labels (A410), and optical trapping was applied in the measurement of antigens and in the study of antibody-antigen interactions (A411). Furthermore, atomic force microscopy was explored in several papers as means for immunoassay detection (A412-A414).

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