# 11 Immunochemical Techniques in Biological Monitoring

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## 11.1 INTRODUCTION

Biological monitoring is the assessment of exposure to an agent through the measurement of biomarker(s) that result from contact with the agent(s). Examples are zinc protoporphyrin in blood, levels of which increase with lead exposure because lead inhibits the biosynthesis of heme; protein and DNA adducts of aromatic amines in blood that can both reflect the intensity of exposure and be correlated with the biologically effective dose; antibodies (Abs) produced against low-molecular-weight molecules—some chemicals, although not immunogenic in their own right because of small size and other limitations, may bind to constitutive polymers (such as host proteins) and become immunogenic, causing the production of specific antibody (Ab). Alternatively, such exposures may lead to the production of new antigenic determinants through non-adduct-forming reactions between the agent and selected protein-carrier molecules. Abs can be made to these modified proteins or to the parent hapten-conjugate. In both cases, the Abs may remain in the human system much longer than the toxicant that initiated their development.

Biomarkers have been trichotomized into biomarkers of exposure, effect, and susceptibility [1]. Biomarkers of exposure are defined as biomarkers that quantify the body burden of chemicals or metabolites of the initial xenobiotic. Biomarkers of effect detect functional change in the biological system under study. Biomarkers of susceptibility indicate the inter-individual variation based on host-susceptibility or genetic differences that can increase or decrease susceptibility. In some cases, a biomarker may simultaneously be a biomarker of exposure, effect, and susceptibility (such as specific IgE in allergies) [2].

When an individual is exposed to a chemical, he or she will receive an internal dose only if the chemical is absorbed into the body. Absorption can occur after dermal contact, inhalation, ingestion, or from a combination of those routes. The extent of absorption from an exposure and the rate of absorption depend on the properties of the chemical (especially its solubility in lipids and water) and the route(s) of exposure [3]. Once absorbed, a chemical is distributed and partitioned into

various tissues as a result of tissue variations in pH, permeability, etc. Highly water-soluble chemicals may be distributed throughout the total body water, whereas more lipophilic substances may concentrate in the body fat or other lipid-rich tissues such as the brain [4]. The loss of chemical from the body can loosely be defined as elimination that depends on metabolism and excretion. Chemicals may be eliminated by numerous routes, including fecal, urinary, exhalation, perspiration, and lactation [5]. A chemical can be excreted from the body without metabolism, in which case, the parent compounds may be detectable in the urine, breath, fecal material, or other body fluid. In other cases, the chemical may be metabolized through oxidation, reduction, hydrolysis, or a combination of these processes, often followed by conjugation with an endogenous substrate [6]. Conjugation of a chemical or metabolite is a pathway for excretion. The more important conjugation reactions include glucuronidation, amino acid conjugation, acetylation, sulfate conjugation, and methylation [4]. Metabolism and excretion and the rates of metabolism and excretion can be affected by age, diet, general health status, race, and other factors. In general, the metabolic products will be more water soluble than the parent chemical [7–10]. Where metabolism yields more than one product, the relative amounts of each and the parent-metabolite ratios are affected by an individual's general health status, diet, genetic makeup, degree of hydration, time after exposure, and other factors.

The kidney is the major organ of excretion and is the primary route for water-soluble substances. These substances enter the urine by either glomerular filtration, tubular secretion, or sometimes both mechanisms. The rate of elimination is directly proportional to the serum chemical or metabolite concentration in first-order elimination [11]. Some xenobiotics such as ethanol have zero order elimination kinetics where, in general, the amount eliminated is independent of its concentration [12]. The relationship between the rate of elimination and serum concentration is linear, and the fraction of xenobiotic eliminated remains constant. The fraction eliminated per unit of time is the elimination rate constant (first order) given by the expression

$$C_t = C_0 e^{(-Kelt)}$$

where $C_t$ is concentration at any time, $C_0 =$ the initial concentration, and Kel = the elimination rate constant. The half-life for elimination ($t_{1/2}$) is the time required for the amount of chemical (plasma concentration) in the body to decrease by half. It is expressed by

$$t_{1/2} = 0.693_{Kel}$$

The percentage of xenobiotic eliminated is (in a first order process) independent of the initial dose; therefore, after about five half-lives, over 95% of the xenobiotic will have been eliminated.

The most common matrices used for biological monitoring are exhaled air, blood, and urine, although saliva, tears, and breast milk can be used [13]. Monitoring exhaled air is limited to body burdens from volatile chemicals. Exhaled air monitoring is not suitable for chemicals inhaled as aerosols or for gases and vapors that decompose upon contact with body fluids or tissues or that are highly soluble in water such as ketones and alcohols [14]. Blood is the medium that transports chemicals and their metabolites in the body. Therefore, most biomarkers present in the body can be found in the blood during some period of time after exposure [14]. A chemical in the blood is in dynamic equilibrium with various parts of the body—the site of entry, tissues where the chemical is stored, and organs where it is metabolized or from which it is excreted. Thus, the concentration of a biomarker in the blood may differ between regions of the circulatory system. This would be the case during pulmonary uptake or elimination of a solvent that would cause differences in concentration between capillary blood (mainly arterial blood) and venous blood. Two advantages of blood monitoring are

1. The gross composition of blood is relatively constant between individuals. This eliminates the need to correct measured biomarker levels for individual differences.
2. Obtaining specimens is straightforward, and with proper care, can be accomplished with relatively little risk of contamination.

An important consideration in blood monitoring is that obtaining blood specimens requires an invasive procedure and should be performed only by trained persons.

Urine is more suitable for monitoring hydrophilic chemicals, metals, and metabolites than for monitoring chemicals poorly soluble in water. The concentration of the biomarker in urine is usually correlated to its mean plasma level during the period the urine dwells in the bladder [15]. In some instances, the urine concentration is affected by the amount of the biomarker stored in the kidneys. Examples are cadmium and chromium. The accuracy of the exposure estimate, using urine monitoring, depends upon the sampling strategy. The most influential factors are time of collection and urine output. Measurements from 24-h specimens are more representative than from spot samples and usually correlate better with intensity of exposure. However, collection, stabilization, and transportation of 24-h specimens in the field are difficult and often not feasible [4]. Determination of biomarkers in individual urine samples is confounded by urine dilution that can vary substantially with fluid intake and physical work load. In practice, this effect of urine dilution is reduced by adjusting the measured concentration of the biomarker to a normal value such as specific gravity [16,17]. This adjustment is made by multiplying the measured concentration of the biomarker by the ratio of [(1.024-1)/(sp.g.-1)] where sp.g. is the specific gravity of the urine sample and 1.024 is the assumed normal specific gravity value. Creatinine concentration is the most frequently used adjustment. Creatinine is excreted by glomerular filtration at a relatively constant rate of 1.0–1.6 g/day. Urinary creatinine concentration can be determined by spectrometric or kinetic methods based on the Jaffé alkaline picrate reaction, enzymatic methods, and methods based on mass spectrometry and liquid chromatography [16]. The adjusted value is the quantity of the biomarker per unit quantity of creatinine. There are other considerations to be taken into account when adjusting urinalysis data for dilution. Adjustment to the creatinine level is not appropriate for compounds such as methanol that are excreted in the kidney primarily by tubular secretion. Because the mechanism of excretion of a biomarker can be altered if the urine is very concentrated or very dilute, measurements on samples, having creatinine concentrations outside the range 0.5–3 g/L or having specific gravities outside the range 1.010–1.030, are unreliable [16]. Adjustment for creatinine concentration, although correcting for dilution, introduces additional variation that must be considered when the data are evaluated. Among the factors affecting the rate of creatinine excretion are the muscularity of the subject, physical activity, urine flow, time of day, diet, pregnancy, and disease [16]. A biological monitoring analytical result is a determination of the level of the biomarker in the biological matrix from which the sample was taken at the time it was taken. Extrapolation from that datum to insight on the exposure of the individual requires knowledge of how the human body responds to the agent. Exposure can be estimated when a quantitative relationship between environmental level and biomarker level has been demonstrated. Health risk can be estimated when a quantitative relationship between a health effect and biomarker level has been demonstrated. Where knowledge of a biomarker is limited, one can only infer from its presence above the background level that exposure has occurred.

For a number of agents there exist published reference levels, termed *biological action levels* by the World Health Organization, that serve as guidelines for interpreting biological monitoring data. In the absence of published biomonitoring action levels, biomarker levels indicating occupational exposure have been inferred by comparison with the normal background levels of the biomarker. Biomonitoring action levels vary in their derivation, some being from correlations with exposure, others with health effects. These reference levels should be used only when one has full understanding of their derivation. When biomarker data are available for exposed and non-exposed populations that are otherwise similar, the upper limit of the range for the non-exposed population

(two or three standard deviations) may serve as a reference level. Levels of biomarker significantly above that limit suggest exposure to the agent. For those biomarkers for which there is no measurable background level in non-exposed humans, this reference level is effectively the detection limit of the analytical method. In any case, levels of the biomarker above the reference level suggest there was exposure, but give no information on the potential health effect.

Biological monitoring data are subject to a number of sources of variability [4,18]. Rates at which an agent is taken up by the body, metabolized, and excreted vary from person to person and are affected by the person's age, sex, physical workload, medications [10], health, and diet [19]. The route of exposure can also affect uptake and metabolism. For example, absorption through the lungs is much faster than absorption through the skin. Thus, the appearance and elimination of a biomarker will be slower if the agent entered through the skin. If the biomarker is rapidly excreted, the optimum timing for collection of biological samples will be different for the two routes of entry. Differing individuals may use differing personal protective equipment and have differing personal work practices. Also, biomarkers can exist in both a free and a conjugated form, the relative proportions of which can vary substantially from person to person. For example, aniline is present in urine as both the free amine and as acetanilide, its acetyl derivative. Some individuals are genetically predisposed to excrete primarily free aniline; whereas others, primarily, acetanilide. There is also the possibility that concurrent exposure to several agents that compete for the same biotransformation sites in the body may occur. This may lead to altered metabolism and excretion that would change the relationship between exposure or health effect and the level of the biomarker [20]. In addition, concurrent exposure to several agents that are metabolized to the same biomarker will be additive. For example, trichloroacetic acid is a biomarker for trichloroethylene, 1,1,1-trichloroethane, and perchloroethylene.

Despite the source of the human specimens used for biological monitoring, acquisition and use of them are covered by federal guidelines (unless exempted) for the use of human specimens (45 CFR part 46- Protection of Human Subjects). These protections include Institutional Review Board (IRB) reviews of protocols involving human subjects with focused attention to informed consent. Depending on the type of biological monitoring measurements performed, analyses may also be impacted by The Clinical Laboratory Improvement Amendments of 1988 (CLIA 88). Key elements of a CLIA program include strict management of specimen collection, handling, storage, and transportation, thus ensuring sample integrity. Some commercial equipment and/or analytical kits used for biological monitoring are Food and Drug Administration (FDA) cleared/approved as in vitro diagnostic devices (IVD) through a process known as premarket notification (510(k) program) that is based on the Medical Device Amendments of 1976 [21]. Many enzyme linked immunosorbent assay (ELISA) assays are 510 K cleared as being essentially equivalent to previously cleared assays. In 1996, the FDA introduced a new IVD classification category called analyte-specific reagents (ASR) [22]. The FDA defines ASRs as "Ab, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens." In essence, the FDA recognized ASRs as the active ingredients of in-house tests, that, when used in combination with general purpose reagents (such as buffers or reactive materials without specific intended uses) and general purpose laboratory instruments, could be the basis for an assay developed and used by a single laboratory. In addition to those tests to which the regulatory oversight of the FDA applies, laboratories may develop and use in-house tests that are not regulated. Such tests may be useful as a tool in the diagnosis of disease; the responsibility for the validation of the test becomes that of the laboratory developing the test. However, there are no rules for validation of these tests. At a minimum, such validation should address evaluation of solid phase binding of Ag or Ab, primary and secondary incubation Ab times, the effect of interfering substances, and matrix effects.

Strict attention to specimen handling and collection is essential for quality data. The analytical laboratory should be consulted for sampling instructions. Analytical methods should provide specific directions on the collection, storage, and transportation of specimens to the laboratory. Adherence to these directions is of the utmost importance to ensure sample integrity. Timing of specimen collection should be appropriate. The method should include instructions for the timing of specimen collection, that is, whether specimens should be obtained during the work shift, at the end of the shift, or at some other time during the work-week. The longer the half-life of the xenobiotic, the less critical is the timing of the collection. The baseline of a biomarker should be evaluated when the toxicant accumulates in the body. The baseline should also be assessed if there is large inter-subject variability in the population. Care should be taken not to contaminate the specimen with either chemicals or bacteria. The proper preservative (for urine or blood samples) or anticoagulant (blood) should be used, when appropriate. Stability of the biomarker is maximized through proper storage and shipment of the specimen to the laboratory and proper storage by the laboratory. When dealing with human specimens, a biosafety program is essential. Pathogens, such as hepatitis B and human immunodeficiency virus (HIV), may be present in blood, saliva, semen, and other body fluids. Transmission can be by an accidental nick with a sharp object, exposure through open cuts, skin abrasions, and even dermatitis or acne and indirectly through contact with a contaminated environmental surface. Engineering controls that include mechanical or physical systems used to eliminate biological hazards must be available. These are items such as biosafety cabinets or self-sheathing needles. Employee work practices are essential to minimize exposure to pathogens. Good personal hygiene procedures and avoidance of needle recapping can lessen exposure to pathogens. Personal protective equipment such as gloves and masks should be used when necessary. Good housekeeping procedures that involve cleanup of the work area are essential to avoid contamination of the laboratory. Employees who have been identified as potential exposure candidates should be vaccinated for hepatitis B. Universal precautions that take into account the above measures should be practiced with every biological sample received. It is not possible to know if a particular sample may contain pathogens; therefore, each sample should be treated as if contaminated.

## 11.2 IMMUNOASSAYS

The classical chemical analysis paradigm used to identify and quantitate an analyte of interest includes isolation of the analyte, separation of the analyte from other potentially interfering substances, and quantitation by instrumental or other methods [23]. These classical methods have many shortcomings, including being highly labor intensive and requiring capital expenditures for expensive equipment, (i.e., gas chromatographs (GC), liquid chromatographs (LC), mass spectrometers (MS), or combinations of these instruments (GC–MS, LC-tandem-MS, etc.). In addition, recoveries during the separation and isolation phases of the paradigm may not be constant, and in some cases, may be associated with the level of analyte in the original sample, potentially yielding confounding systematic errors [24–26]. Despite these shortcomings, however, when adequately controlled, classical chemical biological monitoring has the capacity to quantitate the body burden of substances to the sub-ppb level.

Alternatives to classical chemical analyses are immunoassays. Immunoassays, especially enzyme immunoassays (EIAs) and ELISAs, are commonly used analytical techniques for clinical diagnostic measurements, drug screening, and measurements for evaluating exposure to environmental agents [7,8,23,27–42]. The first ELISA was described in 1971 [43]. Recently, immunoassays have been shown to be useful in evaluating exposure to bioterrorism agents [23,31] such as anthrax. Immunoassays are based on the formation and detection of immune complexes between antigens (Ags) and Ab. Ag are principally macromolecules (proteins, polysaccharides, nucleic acids) that can act as complete immunogens able to stimulate an immune response. Other substances that are too small to act as immunogens on their own (drugs, pesticides,

etc.) have to be coupled to a macromolecular carrier molecule (usually a protein) to become immunogenic and elicit an immune response. These small molecules are called haptens. Many environmental agents (such as pesticides or pesticide metabolites) are haptens. The selection of the protein carrier used to form the hapten-protein conjugated immunogen is important (keyhole limpet hemocyanin, (KLH), a protein from the shelled keyhole limpet, is often used as a carrier protein as vertebrate exposure is unlikely) [44]. The number of haptens bound to the carrier, the chemistry of the conjugation reactions as well as other factors will all impact the final affinity and avidity of the resultant Abs. The purity of the hapten is also important as conjugation of closely related structures to the carrier may result in the formation of non-specific Ab. Spacer molecules are often used in preparation of haptens for conjugation [42] to attempt to increase the specificity of the Ab for the hapten portion of the conjugate. The ability of an Ab molecule to bind an Ag or a hapten is specifically controlled by structural and chemical interactions between the ligand and the Ab at the combining site [45]. The Ag–Ab interaction is reversible and does not involve formation of covalent bonds [45]. This interaction is controlled by the law of mass action

$$\mathrm{Ag} + \mathrm{Ab} = \mathrm{AgAb}$$

and

$$K = \frac{\mathrm{AgAb}}{[\mathrm{Ag}][\mathrm{Ab}]}\ \mathrm{mol}^{-1}$$

where $K$, the affinity constant and AgAb, the Ag-Ab complex. High affinity constants, resulting from stronger Ag/Ab interactions, lead to lower limits of detection (LOD) in immunoassays.

The mammalian immune system has the capacity to produce five distinct classes of Ab (IgA, IgD, IgE, IgG, IgM). Immunoglobulins consist of two identical heavy chains (50–60 kDa) and two light chains (~25 kDa). Both the heavy and light chains have a variable region ($V_H$ and $V_L$) whose sequence varies between Abs. The variable region is the portion where Ag binding occurs. The remainder of both chains is referred to as the constant region ($C_H$ and $C_L$) because it has minimal variation in its amino acid sequence. This variation, however, distinguishes the two light chain subtypes ($\kappa$ and $\lambda$) and the five heavy chain subisotypes ($\alpha$, $\delta$, $\gamma$, $\varepsilon$, and $\mu$). Portions of the constant region are where the Ab binds to cells.

IgG (Figure 11.1) is the preponderant Ab class in most mammals, and as such, is the major Ab used in the development of EIAs. The Ab used in an ELISA can be polyclonal or monoclonal. Polyclonal Abs are usually prepared by injecting animals (usually rabbits) with Ag and adjuvant (a mixture that stimulates the immune response) and then collecting serum from the animals [6]. Polyclonal Abs may be further purified and isolated, yielding essentially monospecific polyclonal Abs [46]. Polyclonal Abs, as the name implies, are a mixture of immunoglobulins directed against specific epitopes present in an Ag (an epitope is the smallest fragment of an Ag to which an immune response can be directed; Ag can have numerous epitopes). The Ab response to each epitope is the result of clonal expansion of specific epitope directed B-lymphocytes.

Monoclonal Abs are produced by fusing tumor cells with cells that produce Ab (hybridoma). Hybridoma cells produce Ab to essentially one epitope, hence the name *monoclonal*. Monoclonal Abs provide a continuous and unlimited supply of a standardized reagent with defined specificity and assay characteristics [47].

Radioimmunoassays (RIA) use radiolabeled (e.g., iodine 125 ($^{125}$I)) reagents that detect the reaction between Ag and Ab. The presence of Ag-Ab reactions are measured using a gamma counter [48]. Most RIA have been replaced by ELISAs, sometimes referred to as EIA. In ELISAs, the solid support (usually a microtiter plate although other solid supports such as magnetic particles, microspheres, coated tubes, etc. have been used) binding of a reactant allows for separation of bound vs. unbound reactants by simple washing. The detector system in ELISAs is usually

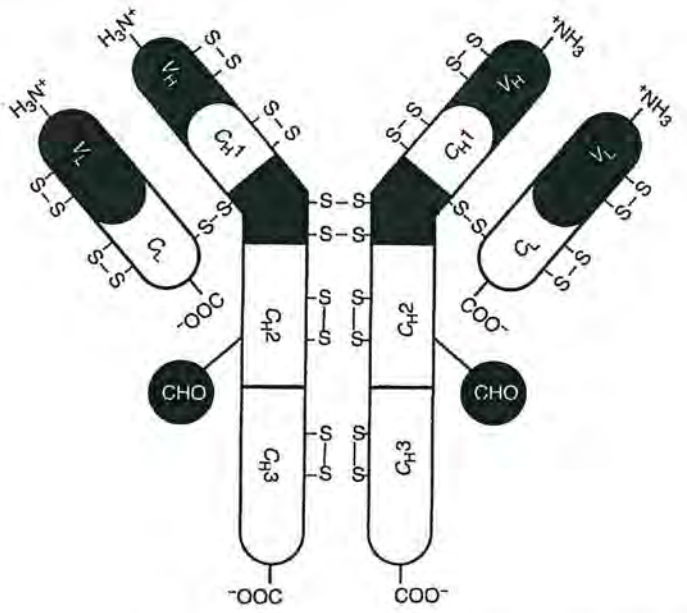


**FIGURE 11.1** Structure of immunoglobulin G (IgG). IgG consists of two identical heavy chains (50–60 kDa) and light chains (~25 kDa). Both the heavy and light chains have variable regions ($V_H$ and $V_L$) whose sequences vary between antibodies. The variable region is the portion where Ag binding occurs. The remainder of both chains is referred to as the constant region ($C_H$ and $C_L$).

an enzyme (e.g., horseradish peroxidase, alkaline phosphatase) bound to a reactant (an Ab or analyte). Common chromogens (enzyme substrates) used in ELISAs include *p*-nitrophenyl phosphate and 2,2′-azino-di-(3-ethylbenz-thiazoline sulfonic acid, *o*-phenylenediamine, and tetramethylbenzidine).

ELISAs can be performed in many different formats (direct, indirect, capture, competitive, etc.). In the following descriptions, generic overviews of ELISA formats are given. Many differing variations of these generic formats have been utilized to detect numerous analytes, all of which would be too exhaustive for the present review. In a direct ELISA (Figure 11.2), the most basic ELISA format, an analyte (hapten, Ab, Ag) is attached to a solid support. Ab, specific for the analyte and containing a reporter system (usually an enzyme), is incubated with the captured analyte. After washing, a chromogen (enzyme substrate) is added and allowed to react, forming a colored product. In an indirect ELISA (Figure 11.2), analyte (hapten, Ab, Ag) is again attached to a solid support. A primary Ab, specific for the analyte, is incubated in the system and the excess removed by washing. A secondary labeled Ab, specific for the primary Ab, is added to the system and incubated. After washing, chromogen is added and the color measured in a spectrophotometer or other instrument. The amount of color produced is proportional to the amount of secondary Ab that was bound. ELISAs may also be designed in capture formats (Figure 11.3). In an Ag capture (sometimes called sandwich) ELISA, Ag is captured by Ag specific Ab that has been attached to the solid support. After washing, another labeled Ab, specific for another epitope on the Ag, is added. After incubation and washing, chromogen is added and the resultant color measured in a spectrophotometer. ELISAs may also be designed as Ab capture ELISAs that are performed in a similar fashion to Ag capture ELISAs, except that the analyte of interest is an Ab. Another format of ELISA is the competitive ELISA. In a competitive ELISA (Figure 11.3), the analyte (either Ab or

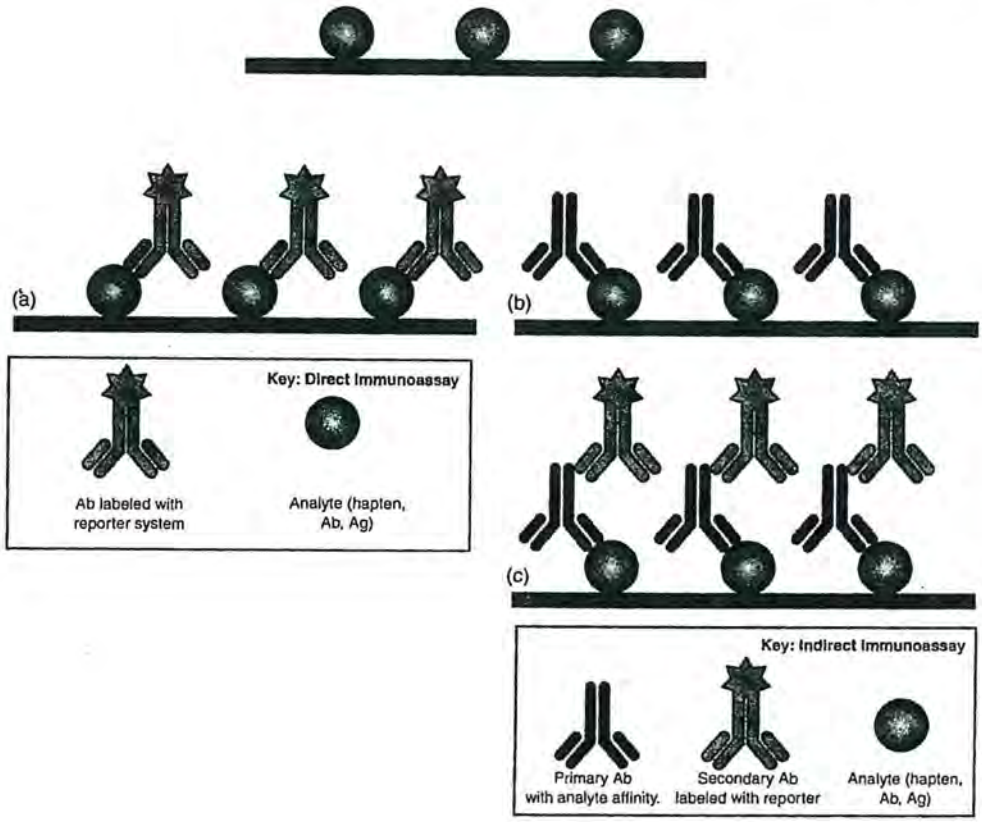


**FIGURE 11.2** Direct and indirect immunoassay. In a direct assay (a), analyte (hapten, Ab, Ag) is bound to a solid support (i.e., bead or microplate). Reporter labeled Ab is introduced to the immobilized analyte, forming an Ag-Ab complex. After washing, the concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system. In an indirect format (b, c), a primary Ab specific for the analyte is introduced to the solid support bound analyte. After washing, a secondary labeled Ab, specific for the primary Ab, is added to the system. The concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system.

Ag) competes with labeled analyte for binding. With higher concentrations of analyte, less of the labeled analyte is bound, yielding a reduced signal. In a modification of this format (blocking ELISA), unlabeled analyte is added prior to the addition of labeled analyte. In most ELISAs, Ag/Ab is coated onto microwell plates by electrostatic attraction and van der Waals forces. Ag or Ab is diluted in coating buffers to assist in immobilizing them to the microplate. Commonly used coating solutions are sodium carbonate, Tris-HCl, and phosphate buffered saline. In order to minimize nonspecific binding to the microtiter plates, solutions of proteins are used to block unbound sites. Commonly used blocking agents are bovine serum albumin, nonfat dry milk, casein, etc.

Body burdens from exposures to pesticides can be estimated from urinary analyses of pesticide parent/metabolites concentrations [9,49–53]. Pesticide applicators as well as others are often exposed to numerous unrelated pesticides, either sequentially or simultaneously. Classically, body burdens of pesticides are analyzed using chemical/instrumental analysis (CIM) or EIAs. Both of these technologies can usually be used to quantitate one analyte (or closely related groups of analytes in CIM) per assay. In addition, CIM assays usually need numerous cleanup and extraction steps before the sample can be introduced to the instrumentation. For example, the

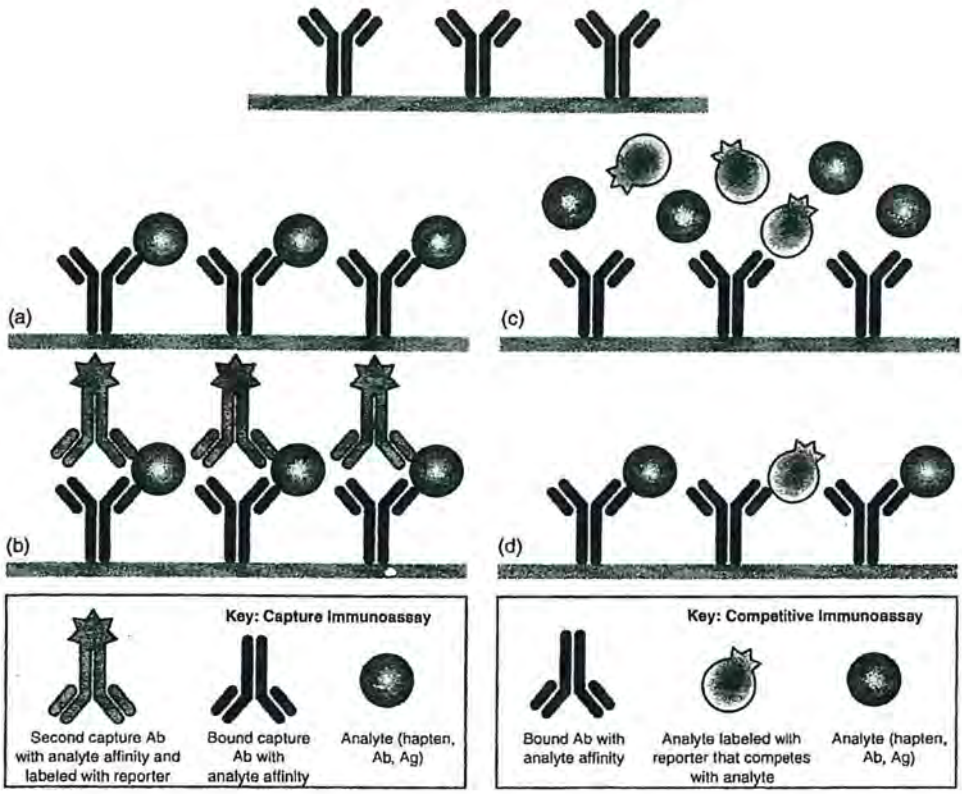


**FIGURE 11.3** Capture and competitive immunoassay. In an Ag capture (sometimes called sandwich) assay, Ab, specific for the analyte, is bound to a solid support. Added analyte is bound by the first specific Ab (a). After washing, another labeled Ab, specific for another epitope on the Ag, is added (b). Concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system. In a competitive assay, analyte and a reporter labeled analyte are allowed to compete for binding sites with the immobilized antibodies (c) and bind in relation to their relative concentrations (d). The concentration of analyte is measured by radiometric, colorimetric, or fluorometric detection of the reporter system. With higher concentrations of analyte, less of the labeled analyte is bound yielding reduced signal.

NIOSH Manual of Analytical Methods (NMAM) method for triazine herbicides and their metabolites in urine [54], using gas chromatography with a mass selective detector, has 39 steps from sample preparation to calculations. To perform multiple analyses for multiple unrelated pesticides by CIM could entail a prodigious amount of effort.

Alternatively, multiple analytes can be simultaneously measured using a multiplexed fluorescence covalent microbead immunoassay (FCMIA). In one example [23] of this method, three distinct spectrally addressable microspheres were coupled with three pesticide conjugates (glyphosate-ovalbumin, atrazine-bovine serum albumin, and metolachlor mercapturate-keyhole limpet hemocyanin) using 1-ethyl-3-(3 dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS). The primary Abs were anti-atrazine, anti-glyphosate, and anti-metolachlor mercapturate. To prepare standard curves, mixtures of atrazine, glyphosate, and metolachlor mercapturate were mixed with the conjugated microspheres and a mixture of primary Ab added. After a period of incubation, biotin labeled anti-rabbit IgG was added

and allowed to incubate. After washing, streptavidin R-phycoerythrin was added and, after incubation and washing, the bead mixture was analyzed in a commercial instrument (see Figure 11.4). This type of assay is basically numerous competitive immunoassays being simultaneously performed using microspheres as solid supports. As the concentration of analyte increases, the reporter signal decreases. In this sytem, 5.6 μm polystyrene, divinyl benzene, and methacrylic acid, microspheres that have surface carboxylate functionalities, were used. Internally, the microspheres are dyed with red and infrared-emitting fluorochromes. The internal concentrations of each fluorochrome are proportioned such that spectrally addressable microsphere sets are obtained. Different Ags are covalently coupled to individual microsphere sets. When the microsphere sets are mixed, they can be analyzed with a standard bench-top flow cytometer or a commercially available

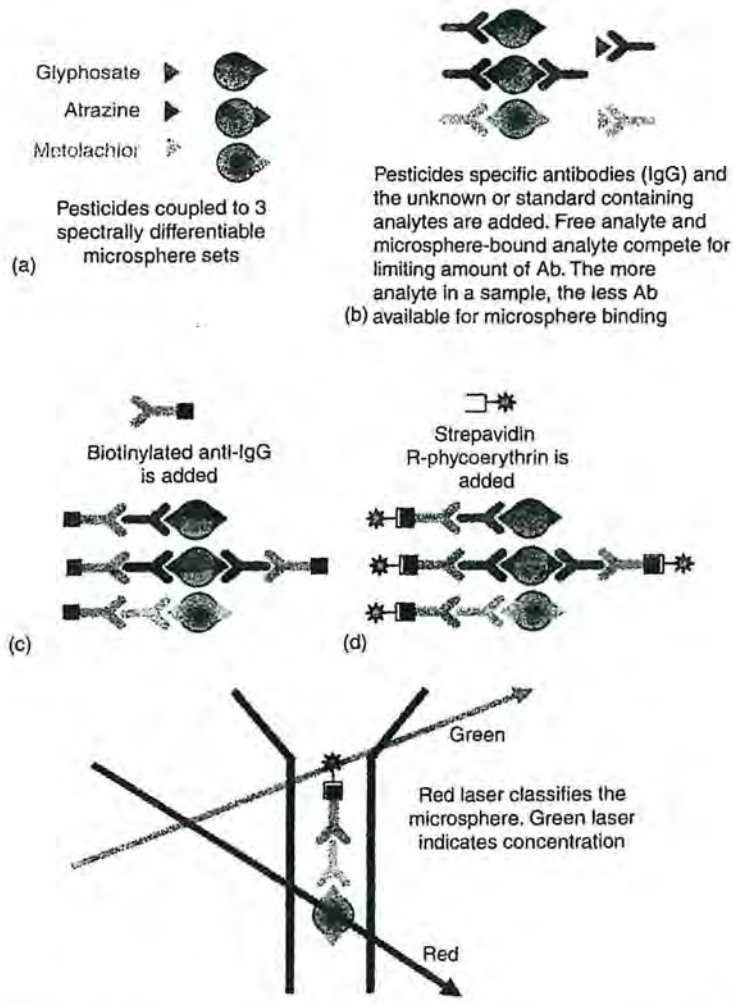


**FIGURE 11.4** Diagram of a competitive multiplexed fluorescence covalent microbead immunoassay (FCMIA). (a) Pesticides coupled to spectrally differentiable microspheres. (b) Bound and free pesticide compete for limiting amount of Ab. (c) Bound Ab is reacted with biotinylated secondary Ab. (d) Reporter (streptavidin-phycoerythrin) is bound and the complex read in a flow cytometer. Red laser indicates bead bound while green laser indicates concentration.

dedicated instrument (Luminex, Inc., Austin, TX). The three major components of the system are a bench-top flow cytometer, microspheres, and computer hardware and software. The flow cytometer analyzes individual microspheres by size and fluorescence, distinguishing three fluorescent colors—green (530 nm), infrared (585 nm), and red (>650 nm)—simultaneously. Microsphere size, determined by 90-degree light scatter, is used to eliminate microsphere aggregates from the analysis. Infrared and red fluorescence are used for microsphere classification, and green fluorescence is used for analyte measurement [55].

The microspheres in a liquid suspension array technique can be conjugated with receptors [56], oligonucleotides [57], proteins [23,30], and Ab [58] such that studies of numerous biological interactions and assays can be performed [49,55,59–61]. In addition, the reporter signal from a FCMIA can be amplified by rolling circle DNA amplification. This is done by covalently attaching an oligonucleotide primer to the reporter Ab in the presence of circular DNA, DNA polymerase, and nucleotides. Amplification results in a long DNA molecule containing hundreds of copies of the circular DNA sequence that remain attached to the Ab. The amplified product is labeled in situ by hybridization with fluor-labeled oligonucleotides [62].

## 11.3 DATA ANALYSIS

Data can be analyzed from ELISA experiments where a set of known standard concentrations of analyte are measured and a relationship between the standards concentration and the ELISA system's response analyzed. This relationship, known as a standard curve, allows one to subsequently estimate the concentration of unknown samples. Many mathematical models have been used to construct ELISA standard curves, including logistic-log transforms [63], log–log transforms [64], four-parameter logistic–log curves, etc. [33]. The four-parameter logistic-log model (4-PL)

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B}$$

where $y$ is the response (optical density), $x$ the analyte concentration, $A$ and $D$ the responses at zero and infinite dose, $C$ the $IC_{50}$ (the concentration giving 50% inhibition), and $B$ a slope parameter [65] has been shown to be superior to log-log and other fits for immunoassays, even when $R^2$ values are high (>0.97). The 4-PL fit extends the range of the assay, thus providing a more precise measurement of analyte concentrations [66].

A practical method for assessing the quality of a standard curve fit is to calculate the concentrations of the standards after the regression has been completed [67,68]. This procedure is also known as standards recovery, and it is performed by calculating the concentration of each standard and then comparing it to the actual concentration using the formula

$$\frac{\text{Observed concentration from the 4-PL fit}}{\text{Expected concentration from analyte added}} \times 100$$

This method yields information about the relative error in the calculation of samples. It is most desirable to have each standard fall between 70 and 130% of the actual value, although more stringent ranges may be applied if greater accuracy is desired. The limitation of using back calculation as the sole method of evaluating goodness of fit is the existence of a bias toward the concentrations of the standards. More specifically, only the standard concentrations are used to assess the quality of the fit; the portions of the curve between each of the standard points are ignored [67]. Spiked recovery may also be used to assess the overall accuracy of an assay [69]. This method incorporates variables in assay preparation as well as the regression analysis. Samples are spiked

with known concentrations of analyte and then analyzed to determine the closeness of the calculated value to the actual value. The chosen concentrations are usually between the concentrations of the standards, thus removing the bias inherent to the back calculation of standards method. The results are assessed in the same manner as the standards recovery, using the formula above. A spiked recovery value between 80 and 120% is considered acceptable. The disadvantage of this method is that it is affected by variables other than curve fitting. Errors in sample preparation or assay preparation (pipetting, adding reagents) may affect overall recovery. In addition, it is difficult to accurately spike low levels of analyte into samples because of the relative imprecision of pipets that deliver small volumes [70].

Estimates of least detectable doses (LDD) and minimum detectable concentrations (MDC) have also been calculated using numerous methods, including graphically from the intersection of the asymptote of a 4-PL regression's 95% confidence interval (CI) with the regression line [71] or as a multiple of standard deviations of the blank response [72,73]. In competitive assays where the response is expressed as $\%B/B_0$ data with $B =$ to the response of a standard and $B_0 =$ to the mean optical density measured for the blank, $90\%B/B_0$ is routinely used as the LDD [74].

Specificity is an important characteristic of any laboratory test, describing its ability to distinguish between true (or specific) and non-specific results. With immunoassay methods, interferences that affect specificity can be categorized into two major classes: (1) those that affect the binding event between the Ab and an Ag in a general way such as pH or ionic strength or (2) those substances that affect binding of Ag by competing for the specific binding site on the Ab. These specific interferences are often referred to as cross-reactants. In the analysis of pesticides and/or pesticide metabolites, it is often desirable to have high levels of cross-reactivity with related compounds and metabolites of the parent compounds so that broad screening can be performed. The specificity of an immunoassay may be characterized by adding increasing amounts of a potential cross-reacting substance to a sample and measuring the response in the immunoassay. The results of this experiment can be reported in several ways. One method of representing the comparative reactivity of these compounds is to determine the concentration of each compound required to displace the same amount of labeled Ag from Ab. For example, one commonly calculates cross-reactivity using the concentrations required to displace 50% of the label or 50% $B/B_0$. The concentration is called the $ED_{50}$ (estimated dose at 50% $B/B_0$). A ratio of the resulting concentrations can be referred to as the "percent cross-reactivity at the $ED_{50}$." Cross-reactivity can also be calculated at other levels of displacement such as 20% ($ED_{20}$). Depending on the slope and shape of the response curve, the % cross-reactivity may be different at different displacement levels. Another method to report cross-reactivity may be to simply report the concentration of cross-reactant required to displace a given amount of labeled Ag. For example, one might report the concentration of cross-reactant required to displace 50% of the label (i.e., the $ED_{50}$). Again, different displacement levels can be used, but the absolute result and, possibly, the relative results will change. If one chooses the lowest level of displacement that can be reliably distinguished from zero displacement, the resulting concentrations could be represented as a LDD for each cross-reactant. Evaluation of cross-reactivity in poorly defined biological samples may be very complex [75].

Numerous extraneous factors can be present in a sample that may influence Ag-Ab binding, including pH, ionic strength, endogenous components such as enzymes, immunoglobulins, bile, and salts, and exogenous substances such as drugs, polymers, and detergents [76]. These factors contribute to matrix effects defined as follows: the influence of a sample property, other than analyte, on the measurement, and thereby on the measured values; and the physicochemical effect(s) of the matrix on the analytical method's ability to accurately measure an analyte [76].

To insure the integrity of immunoassay data, analytical quality control measures are an important component. Each analyst must take an independent responsibility for assuring that the analytical quality control system works. This can be accomplished by using known spiked samples that closely simulate the samples being analyzed with regard to concentration and interferences.

Because the analyst is most familiar with the methods being used and should know what range of recoveries to expect, problems with the system can be detected early. At a minimum, the following have to be evaluated [77]:

1. Blanks: Analyte free buffer or water.
2. Standards: Calibration curves should be prepared with triplicate points at each of at least five different concentration levels that bracket the concentration of actual samples to avoid extrapolation. Standards should be prepared in the same diluent as used for samples.
3. Blind Spiked Samples: Blind spiked samples are prepared by someone other than the analyst performing the measurement and are to provide an independent check on the accuracy and precision of the measurement.
4. Precision Analysis: Intra-assay and interassay coefficients of variation should be calculated, and their trends evaluated using quality control charting.

## 11.4 PESTICIDES

The United States Environmental Protection Agency estimates that 10,000–20,000 physician-diagnosed pesticide poisonings occur each year among the approximately 3,380,000 U.S. agricultural workers [78]. The Centers for Disease Control and Prevention, in their Second National Report on Human Exposure to Environmental Chemicals, measured urinary levels of pesticide parent/metabolites for organochlorines, organophosphates, carbamates, and herbicides as part of a report of biomonitoring exposure data for 116 environmental chemicals for the non-institutionalized, civilian U.S. population over the 2-year period 1999–2000. Results from that report showed results greater than the LODs for the majority of pesticide parent/metabolites measured [79]. Exposure to low doses of pesticide mixtures is thought to be related to chronic health effects in humans [80]. Human exposure to pesticides is multi-media and multi-route. Agricultural workers can be exposed to numerous pesticides for variable periods of time, at variable exposure levels, and by numerous routes (inhalation, dermal, ingestion). In addition, transfer exposures can occur from dermal or other contact with contaminated equipment and surfaces. Primary and transfer exposures are affected by weather conditions, type of applications, and work practices [6,9,10,30]. Estimates of pesticide exposures to equipment or clothing may be performed by analytical chemical analyses of elutions [81,82], whereas body burdens of pesticides are usually estimated by biological monitoring of urine samples [6,7,9]. EIAs have been used to measure pesticide concentrations in surface, rain, and groundwater for a variety of substances including alachlor, amitrole, atrazine, bentazon, bromacil, chlorodiamino-*s*-triazine, chlorsulfuron, clomazone, cyanazine, diethylatrazine, diclofop-methyl, 2,4-D, dichlorprop, diuron, hexazinone, hydroxyatrazine, imazamethabenz, imazaquin, isoproturon, maleic hydrazide, MCPB, metazachlor, methabenzthiazuron, metolachlor, molinate, monuron, norflurazon, paraquat, picloram, propazine, simazine, terbuthylazine, terbutryn, thiobencarb, triasulfuron, 2,4,5-T, trifluralin, fenitrothion, chlorpyrifos, heptachlor, methoprene, 1-naphthol, parathion, paraoxon, PCP, permethrin, pyrimiphos-methyl, quassin, 3,5,6-trichloro-2-pyridinol, benomyl, benzimidazole captan, chlorothalonil, fenpropimorph, iprodione, metalaxyl, myclobutanil, procymidone thiabendazole, triadimefon, and triazole [45]. The majority of these analyses were for parent compounds.

Urinary EIAs have been used to estimate body burdens of numerous pesticides [7,9,10,30,33,34,36,39,40,49–53,74,83–88]. Many commercial suppliers offer EIA kits for the measurement of pesticides in water and other matrices (e.g., EnviroLogix Inc., Portland, ME; Strategic Diagnostics Inc., Newark, DE; Abraxis LLC, Warminster, PA). EIA test kits are ideally suited when speed, simplicity, sensitivity, and low cost are important criteria. Immunoassays are appropriate when specific chemicals, or families of chemicals, are known or suspect, and

the objective is to determine their presence, absence, or quantity contained within the sample. In some cases, commercial EIA kits, designed primarily to measure pesticide parent, can be used to screen for urinary metabolites. This is due to the apparent cross-reactivity of the Ab used in the commercial kit for the parent compound, also having affinity for the metabolite. For example, alachlor, MW 270, is too small to be immunogenic in its own right. To overcome this, most Ab for alachlor and other chloroacetanilide herbicides are raised against a derivatized chloroacetanilide that is coupled to a carrier macromolecule (usually a protein), forming a thioether linkage [89]. Polyclonal antisera to these alachlor-protein-thioethers would be expected to contain Ab to numerous antigenic determinants on the immunogen molecule, including the thioether region, probably with differing affinities and avidities for each antigenic determinant. Alachlor is metabolized to a mercapturate metabolite [90] that cross-reacts with some commercial Ab, actually showing an approximate 4x greater affinity than that shown for parent [7].

Commercial EIA test kits specifically designed for human pesticide exposure monitoring are also available. EnviroLogix Inc., Portland, ME offers test kits for the measurement of alachlor mercapturate, atrazine mercapturate, *N,N*-diethyl-*m*-toluamide (DEET), and metolachlor mercapturate in urine. These kits can be fast, accurate, and precise.

Conventional analytical techniques, including both classical instrumental methods and EIAs, although highly precise and accurate, are in reality, laboratory-based techniques [91]. Immunobiosensors (analytical devices with the potential for portability that combine the specificity of Ag–Ab interaction with a transducer that produces a signal proportional to the target analyte concentration) have been described [92]. The Ag-Ab complex is in close contact with a signal transducer (e.g., optical, amperometric, potentiometric, or acoustic) coupled to a data acquisition and processing system [93]. Immunobiosensors, because of their specificity, fast response times, low cost, portability, ease of use, and continuous real time signal, present distinct advantages over alternative methods of analyses [94].

The primary optical characteristics that are exploited in the development of immunobiosensors are fluorescence, chemiluminescence, and refractive index change. These optical effects can be measured by surface plasmon resonance (SPR) or evanescent wave effects [95,96]. For example, fluorescent fiber optic biosensors have optical fiber probes, each coated with an Ab specific for a particular analyte. Samples flow over the probes, followed by another Ab that has been attached to a fluorescent dye. If the fluorescent Ab binds to the captured agent, a fluorescent signal is generated at the surface of the probe (Figure 11.5).

Refractive index (the bending of light at the interface of two media) can also be exploited to measure Ag-Ab interactions. The way light interacts at an interface can be exploited to measure changes in surface conditions as occur when Ag binds to Ab on a surface and may be measured by SPR [97] (Figure 11.6). An SPR immunobiosensor is composed of an SPR transducer and a biological recognition element (e.g., Ab) that recognizes and is able to interact with the targeted analyte. The biomolecular recognition element is immobilized on the SPR transducer surface. When a liquid sample is brought in contact with the sensor surface, the interaction between the biomolecular recognition element and the analyte occurs, producing a change in the refractive index at the sensor surface. This in turn results in a change in the propagation constant of a surface plasmon excited at the sensor surface, and it is eventually measured by measuring a change in one of the characteristics of light interacting with the surface plasmon—resonant wavelength, resonant angle, intensity, phase, and polarization.

Piezoelectric immunobiosensors are based on a quartz crystal resonator (Figure 11.7), consisting of a disk with electrodes plated on it. Application of an external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal. The frequency of the vibration can be determined by a frequency counter and is affected by changes in mass associated with Ag binding to the surface-immobilized Ab. This binding increases the mass of the crystal, decreasing the resonant frequency that can be measured [98].

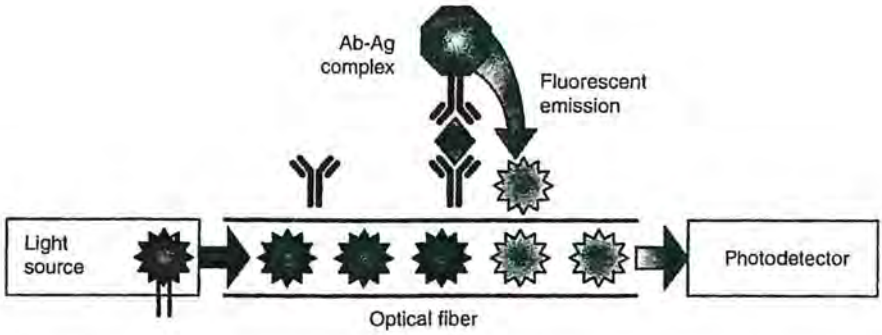


**FIGURE 11.5** Fluorescence labeled biosensors. Fluorochrome molecules are used to label secondary antibodies that bind to the Ag in a sandwich format. The fluorochrome is excited by absorbing short-wavelength light, and then it emits light at a higher wavelength that can be detected by the biosensor transducer.

Light-addressable potentiometric sensors (LAPS) combine both electrochemical and electro-optical detection, measuring small pH differences ($\sim$0.01 pH units) on a semiconductor (Figure 11.8). The pH-sensing region of the instrument consists of a silicon layer wired into an electrical circuit. A LAPS measures an alternating photocurrent, generated when a light source such as a light emitting diode (LED), flashes rapidly. The current magnitude depends on the surface potential that, in turn, depends on the surface pH [99].

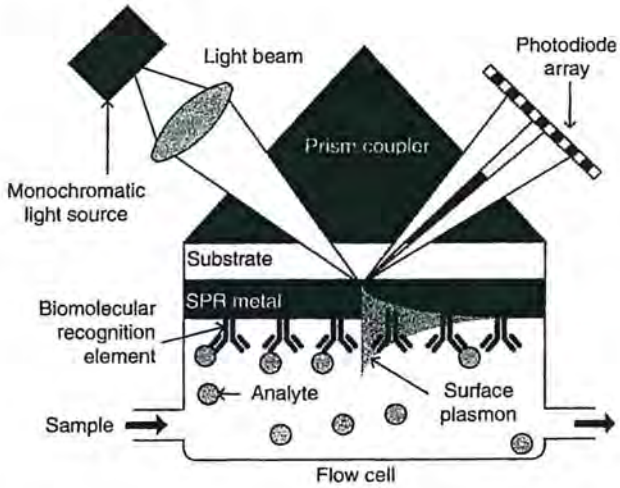


**FIGURE 11.6** Surface Plasmon Resonance (SPR). A narrow-band convergent beam from a light-emitting diode is launched into a prism coupler and made incident onto a thin metal (gold) film. The angular component of light that fulfills the coupling condition excites a surface plasmon wave at the outer boundary of the metal film. The coupling produces a narrow dip in the angular spectrum of the reflected light; the precise angular position is determined using a computer-controlled position sensitive photodetector. When a solution containing analyte molecules is injected into the flow-cell, analyte molecules in the sample bind to the biomolecular recognition elements immobilized on the SPR sensor surface, producing a shift in the position of the dip in the angular spectrum of reflected light. The shift can be correlated with the concentration of analyte in the sample.

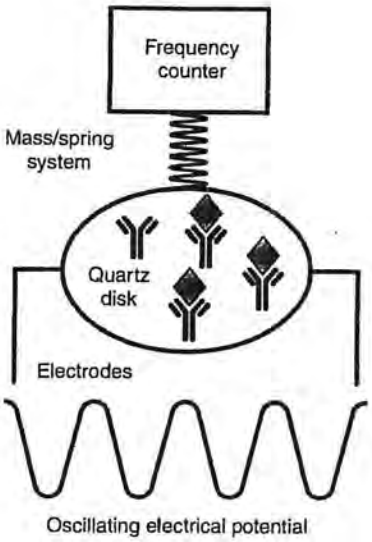


FIGURE 11.7 Piezoelectric biosensors. Piezoelectric (PZ) biosensors are based on a quartz crystal resonator, consisting of a disk with electrodes plated on it. The application of an external oscillating electric potential across the device induces an acoustic wave that propagates through the crystal. The frequency of the vibration can be determined by a frequency counter. The frequency is affected by changes in mass associated with Ag binding to the surface-immobilized Ab. Such binding increases the mass of the crystal, decreasing the resonant frequency of the crystal.

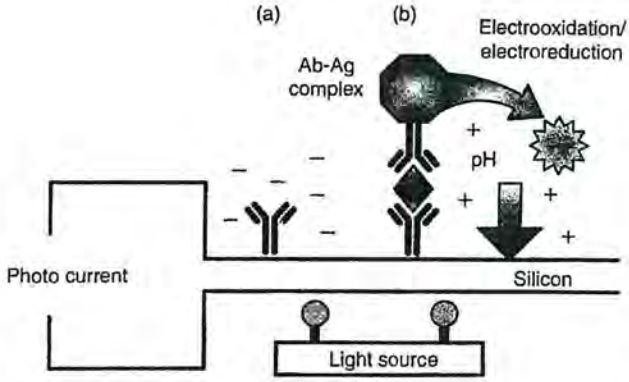


**FIGURE 11.8** LAPS (Light addressable potentiometric sensor). A LAPS combines both electrochemical and electroptical detection, measuring small pH differences on a semiconductor. The pH-sensing region of the LAPS consists of a silicon layer wired into an electrical circuit. An alternating photocurrent is generated by the light source. The magnitude of the current depends on the surface potential that, in turn, depends on the surface pH. (a) No Ag bound to the primary Ab, the potential (−) is the potential generated by the light source. (b) Ag-labeled secondary Ab complex bound to the primary Ab catalyzes electrooxidation/electroreduction, thereby producing a pH change that affects the surface potential (+).

In conclusion, biological monitoring provides the basis for estimating an internal chemical dose by measuring parent compound and/or metabolite concentrations in selected body tissues and fluids. In contrast to biological monitoring that measures the compound or its metabolites in human tissue, biological effects monitoring (i.e., use of biomarkers) is used to detect evidence of chemical exposure by measuring a biochemical response, such as changes in enzyme activity [100]. In other words, chemical exposure is estimated based on an indicator property rather than through direct quantification of the chemical itself. This type of monitoring does not provide a direct measure of internal dose, but it can provide an indication of the potential for adverse effects. Dose cannot be estimated unless the correlation between exposure and biochemical response is well understood.

Biological monitoring has been classically performed by quantitative analyses for urinary-excreted or environmentally sampled chemicals by chemical/instrumental analysis (CIM) after extraction from urine or sample matrices (such as rinses of hands, patches on skin/clothing, sorbent materials, and filters). These procedures are costly, time consuming, labor intensive, and require the acquisition of high capital expenditure equipment and highly trained personnel, although they are usually highly specific. Alternatives to CIM are EIA where pesticides or their metabolites can be quantified in neat or diluted urine or water using Ab (usually polyclonal) directed against the pesticides or their metabolites. EIAs have been used to measure numerous types of analytes in both biological and environmental matrices over the last 35 years. EIAs have the benefit of being inexpensive, fast, and quantitative, and they can be simply performed on relatively inexpensive equipment. In many cases, EIAs have lower limits of quantitation than CIMs. Urinary pesticide/metabolite EIAs may have the disadvantages (in some cases) of not being specific or of suffering from matrix effects from urine, limiting their sensitivity by a factor of 10–100-fold. However, when speed and cost efficiency are evaluated, immunoassays significantly outperform conventional methods. The ability of immunoassays to be multiplexed (the measurement of numerous analytes simultaneously) is one factor that distinguishes EIAs from CIMs. Diversity in the chemical properties of mixtures has been shown to negatively impact recoveries when measuring multiple analytes by CIM [29]. The final outcome of these efforts are methods that are either sensitive and imprecise or precise and insensitive [24,101,102]. The future use of immunobiosensors, surface and liquid matrix arrays, and other cutting edge technologies (especially as these methods mature) for biological monitoring should allow for the almost instantaneous measurement of numerous analytes simultaneously with accuracy and precision.

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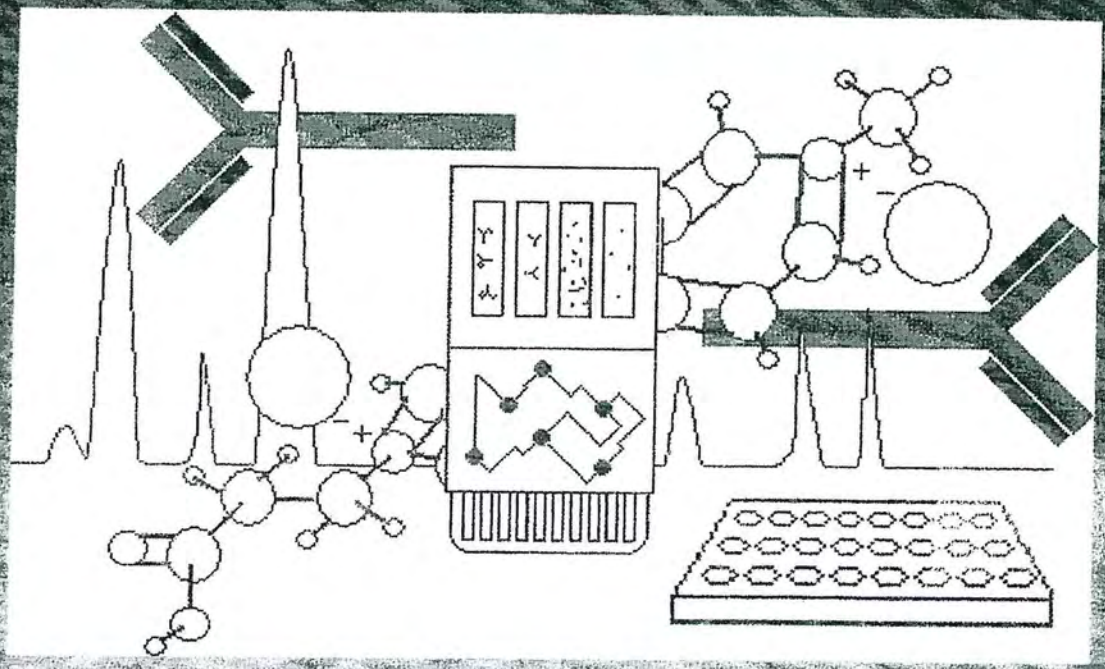

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