20 Multiplexed Immunoassays in Food Analysis

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20.1 INTRODUCTION

In general, immunoassays can be defined as analytical methods in which antibodies are used for the identification and quantification of defined target analytes. At present, immunoassays are the most commonly used types of ligand-binding assays for the identification of a large variety of analytes, such as proteins, peptides, microorganisms, and low molecular weight molecules [1]. Multiplexed immunoassays (simultaneous multianalyte immunoassays), in which several analytes are measured simultaneously in a single assay, present several advantages over single-analyte assays such as reduction of analysis time, work simplification, decrease in total sample volume, and lower overall cost per test [2,3]. The ability to simultaneously measure multiple analytes in a single assay holds
enormous potential for meeting the growing demands of proteomics, diagnosis, environmental monitoring, food safety, and homeland security.

Multiplexed immunoassays are broadly classified as multiple-label assays and spatially resolved assays. Multiple-label assays are methods in which antibodies or antigens are localized in the same assay zone with distinct labels such as radiolabels, fluorophores, enzymes, metals, and nanocrystals. Spatially resolved assays use discrete analytical zones on a single device without the need for multiple labels. The substrates used in spatially separated assays are usually membranes, capillaries, microchips, microtiter plates, beads (microspheres), and electrodes.

20.2 MULTIPLE-LABEL IMMUNOASSAYS

20.2.1 MULTIPLE RADIOISOTOPES

The first multiplexed immunoassays employed multiple radioisotopic labels. Dual-analyte immunoassays reported in 1966 used $^{131}$I and $^{125}$I for the simultaneous detection of human insulin and growth hormone [4]. The discrimination of radioisotopes requires that the energy spectra are distinct. The radioisotopes $^{125}$I and $^{57}$Co have been combined for the detection of lutropin and follitropin [5], vitamin B$_{12}$ and folic acid [6], as well as thyrotropin and thyroxin [7]. Because of the concerns over the safety of radioisotopic labels and the disposal of radioactive waste, non-isotopic labels are typically chosen for immunoassays today.

20.2.2 MULTIPLE LANthanides

Lanthanides are series of elements of increasing atomic numbers beginning with lanthanum (57) or cerium (58) and ending with lutetium (71). Lanthanides for time-resolved fluorescence detection were the first choice of non-isotopic labels for multiplexed immunoassays because of their characteristic narrow-banded emission lines. Their emissions are clearly distinguishable from one another with respect to both wavelengths and lifetimes [8]. In addition, time-resolved fluorescence is detectable at low levels and enables the development of several highly sensitive immunoassays because of the nearly zero background signal [9]. Europium (Eu$^{3+}$) and terbium (Tb$^{3+}$) as well as europium and samarium (Sm$^{3+}$) pairs have been effectively combined for dual-analyte immunoassays [10–13]. Xu et al. [14] even described a quadruple-label fluorometric immunoassay using Eu$^{3+}$, Tb$^{3+}$, Sm$^{3+}$, and Dy$^{3+}$ (dysprosium) for a simultaneous detection of thyroid-stimulating hormone, 17-$\alpha$-hydroxyprogesterone, immunoreactive trypsin, and creatine kinase MM isoenzyme in dried blood spots. The assay was performed with a sandwich format in a microtiter well. The limits of detection (LODs) for thyrotropin, 17-$\alpha$-hydroxyprogesterone, immunoreactive trypsin, and creatine kinase MM isoenzyme were 0.1 mIU/L, 2 nmol/L, 2 µg/L, and 4 U/L, respectively.

20.2.3 MULTIPLE VISIBLE AND FLUORESCENT DYES

Fluorescent dyes are commonly used in single-analyte immunoassays. However, their broad emission spectra with red spectral tails have limited their use in multiple-label assays owing to spectral overlap; therefore, only dual-dye assays were used in multiplexed immunoassays. Green fluorescein and red rhodamine or their derivatives are the two common dyes used for dual-analyte immunoassays [15–17]. Highly colored fluorescent dyes may also be used as visible dyes based on spectral reflectance measurements. A multianalyte lateral-flow immunoassay was developed using three preparations of liposomes, each encapsulating a different dye, which were captured in a single zone on a lateral-flow strip, and the reflectance spectrum was measured. Partial least square regression was used to develop multivariate calibrations that could successfully deconvolute the overlapping spectra [18].
20.2.4 MULTIPLE ENZYMES

The most commonly used labels for immunoassays are enzymes, of which ELISAs (enzyme-linked immunosorbent assays) are the primary format. Both enzyme-labeled and substrate-labeled fluorescent immunoassays have been used for dual-analyte detection. Alkaline phosphatase, horseradish peroxidase, and β-galactosidase are the three most popular enzymes for immunoassays. The combination of alkaline phosphatase and β-galactosidase was used for the detection of two thyroid hormones [19]. However, enzyme labels usually have different optimum ranges of temperature, pH, and reaction time for their activity. For example, the optimum pH for alkaline phosphatase, horseradish peroxidase, and β-galactosidase are 8–10, 5–7, and 6–8, respectively [20]. Therefore, identifying a final set of assay conditions without sacrificing sensitivity would be a difficult task. To eliminate the difficulty, bound enzymes were sequentially reacted with the corresponding substrates [21–24]. To avoid the problem that results from using the same kind of label, an enzyme was also combined with radioisotope in a dual-analyte immunoassay [25].

20.2.5 MULTIPLE METALS

Different metals have been used with different transducers for multi-label immunoassays. Salmain et al. [26] used FTIR (Fourier transform infrared) spectroscopy to simultaneously detect two distinct organometallic labels (metal carbonyl complexes). Hayes et al. [27] used differential pulse anodic stripping voltammetry at a hanging mercury drop electrode to detect two metal ion labels (In³⁺ and Bi³⁺). Bordes et al. [28] developed a dual-analyte immunoassay using two cationic metal complexes (cobaltocene and ferroceneammonium) and a Nafion-loaded carbon paste electrode by square-wave voltammetry. Although they later also added pentaammine ruthenium(II) complexes for a simultaneous detection of three-labeled drugs, the immunoassay was not successful due to the instability of the pentaammine ruthenium(II) complexes [29].

20.2.6 MULTIPLE NANOCRYSTALS

Inorganic nanocrystals are relatively new classes of labels for immunoassays. Luminescent, colloidal, and semiconductor nanocrystals (quantum dots, QDs) have the potential to overcome the limitations of organic fluorophores. They have broad excitation as well as size-dependent, tunable, narrow-emission spectra that allow the simultaneous excitation of several different-colored QDs at a single wavelength with little spectral emission overlap for multiplex analysis [30–32]. Also, QDs have been reported to be about 20 times brighter and 100 times more photostable in comparison with organic dyes such as rhodamine [33]. QDs enable multiplex assays, requiring only a single excitation source having an excitation wavelength far from that of the QD emission peaks [29]. Goldman et al. [29] used four antibody-conjugated QDs of different sizes to demonstrate multiplexed assays for four protein toxins in a single sample. The LODs for the detection of cholera toxin, ricin, shiga-like toxin 1, and staphylococcal enterotoxin B were 10, 30, 300, and 3 ng/mL, respectively. However, emission spectral overlaps were still observed in the four QDs even though they have relative narrow-emission spectra. A linear equation-based algorithm was used to deconvolute the signal from mixed toxin samples for the simultaneous quantitation of all four toxins.

Nanocrystals are not only ideal optical labels, but also excellent electrochemical labels for immunoassays. Liu et al. [34] detected four proteins (β-microglobulin, IgG, bovine serum albumin, and C-reactive protein) simultaneously using four kinds of colloidal nanocrystals (ZnS, CdS, PbS, and CuS) yielding distinct voltammetric peaks, the positions and sizes of which reflect the identity and concentration of the respective analytes. Nanocrystals were measured at an in-situ plated mercury film on a glassy carbon electrode by square-wave anodic stripping voltammetry, which combines the amplification feature of stripping voltammetry with the speed advantage of square-wave scanning. Femtomole detection limits resulted from the combination of electrochemical stripping transduction and immunomagnetic beads.
20.3 SPATIALLY RESOLVED MULTIPLEXED IMMUNOASSAYS

20.3.1 Membrane Assays

Membranes have been the standard substrate for western blot assays. Western blotting first separates antigens electrophoretically on a gel, and then a membrane (usually nitrocellulose) is placed on the gel and antigen bands are driven onto the membrane using electrophoresis. The membrane, which provides discrete spatially separated test zones, is then incubated with antibodies. Combined with multiple labels (usually enzymes), a multiplexed detection is achieved [35]. Antigens can also be applied directly to the membrane in parallel lines. Ijsselmuiden et al. [36] used this technique to detect antibodies against two treponemal antigens. A disposable membrane test card with multiple test areas processed in an incubation instrument for simultaneous determinations from a single specimen was designed by Donohue et al. [37]. Buechler et al. [38] used the same principle with colloidal gold labels to simultaneously detect seven drugs of abuse in urine.

Instead of incubating the whole membrane card with samples and reagents, an alternative assay format is used to apply the sample and reagents at one end of a membrane strip, and the mixture moves along the membrane to multiple analytical zones by capillary migration (Figure 20.1). This type of membrane strip assay is called an immunochromatographic dipstick or a lateral-flow assay. The advantage of this type of assay is simple handling without requiring any washing steps, thereby usually being completed in less than 30 min. The intensities of the color bands, which correlate to the concentrations of the analytes, can be visually estimated or quantified by a reflectometer [39], a microarray scanner [40], or densitometry using specialized computer software [41]. This type of assay has also been applied to the simultaneous detection of drugs of abuse in urine [37]. Several researchers also used this device for multiplexed detection of food contaminants, including pesticide residues [42,43] and mycotoxins [44,45].

20.3.2 Capillary Assays

Capillary-based immunoassays employ flow-through optical systems. Capillaries have several advantages over other substrates for assays, such as improved assay kinetics attributed to the higher surface-to-volume ratio and less mass transport of the reactants being required [46]. Also, consumption of reagents is reduced and samples for the immunoassays are small due to the small dimensions of capillaries [47]. Capillaries can be made of plastic [47,48] and fused silica [46,49]. Antibodies have usually been immobilized in the capillary by physical adsorption [46–48]; however, it has also been reported that antibodies were coated onto the silanized inner walls of fused

![Diagram of Capillary Assay](image-url)

**FIGURE 20.1** Multiplexed lateral-flow assay format. The test strip has multiple discrete analytical zones. Analytes bind to the reagents in the mixture, which migrates through the test strip to reach the analytical zones by capillary action, thereby developing the color in the bands on the test strip.
silica capillaries using a heterobifunctional crosslinker, N-succinimidyl-4-maleimidobutyrate [49]. Platforms of either multiple capillaries coated with different antibodies [46,49] or multiple discrete antibody bands in one capillary [47,48] have been used for the capillary-based multiplexed immunoassays. Petrou et al. [48] used a precision microsyringe to inject 2.5 µL of each antibody solution into the capillary, thereby forming three discrete antibody bands. The multiple fluorescent bands formed in the capillary can be quantified by scanning the capillary with a light beam of appropriate wavelength for the excitation of the fluorescent label (Figure 20.2). The photons are trapped by internal reflectance in the capillary walls and waveguided toward its end. A light sensor detects the waveguided photons at the end of the capillary [48]. Three hormones in human serum, follitropin, human chorionic gonadotropin, and prolactin could be detected with LODs of 1.3 µg/L, 2.3 IU/L, and 3.6 IU/L, respectively. Although they were adequate for the determination of hormones in human serum samples, they were higher than those determined by microtiter plate assays (0.7 µg/L, 1.3 IU/L, and 2.6 IU/L, respectively) [48].

20.3.3 Microchip Assays

Multiplexed immunoassays are currently undergoing intensive development because of the application of protein microarrays (microchip) and proteomics. Microchip technology development has made great advances and has shown the most promising potential for multiplexed immunoassays in terms of the high throughput. A large number of studies have been reported on microchip surface materials, biomolecule patterning, assay formats, and detection systems.

20.3.3.1 Surface Materials

It is ideal for microchips to have a surface material providing high binding capacity, high protein density, low nonspecific background, and high reproducibility. Although PVDF (polyvinylidene fluoride) membranes were commonly used in the early array-based immunoassays [50–52], they did not allow a sufficiently high protein density [53] because of the spreading of the spotted materials. Therefore, glass slides have become the preferred solid supports, which are able to provide 1600
spots/cm² [54]. Glass also has great durability, good optical properties, and compatibility with the platforms already established for DNA microarrays. Since the glass surface is not suitable for passive adsorption of biomolecules, polystyrene [55] and polylysine [56] have been used to modify the glass surface for adsorption. To achieve more specific and stronger biomolecule attachment, glass slides are also activated with coupling groups, such as aldehyde, epoxy, carboxylic esters, mercaptopropyl trimethoxysilane, or amine, to covalently crosslink to biomolecules [57,58]. Polymer layers (dendrimer and polyethylene glycol) with coupling groups have also been used to coat the glass to reduce nonspecific binding and to increase the density of accessible functional groups [59]. An alternative is high-porosity gels or membrane-coated surfaces, such as hydrogel [60,61], agarose [62], and nitrocellulose [63]. These surfaces prevent both rapid evaporation and the close contact of the protein with the surface, thereby preserving the three-dimensional structures of immobilized biomolecules. Also, hydrogels were reported to have low intrinsic background fluorescence that further improves the sensitivity of immunoassays [64]. The polyacrylamide-based hydrogel substrate yielded a sixfold higher signal-to-noise ratio than the poly-L-lysine substrate in an antibody microarray profiling of human prostate cancer sera [65]. Although it has been reported that lower detection limits were achieved, reproducibility was poorer with the hydrogel slides than with non-gel-coated slides [55].

Gold-coating has also been used to integrate surface plasmon resonance (SPR) [66,67] or mass spectrometry [68] as the detection system. In general, a bifunctional thio-alkylene is usually used to spontaneously form a self-assembled monolayer (SAM), which has a SH-group that reacts with the gold surface while the other free end reacts with the bio-recognition molecules.

### 20.3.3.2 Biomolecule Patterning

Many biomolecule patterning methods have been used such as microcontact printing (spotting), ink-jet printing, DNA-directed immobilization (DDI), and microfluidic network (µFN) patterning.

#### 20.3.3.2.1 Microcontact Printing

Microcontact printing (spotting) delivers sub-nanoliter sample volumes directly to the surface using tiny pins that deliver a liquid film onto the surface. It is particularly convenient and simple, requiring no extraordinary apparatus or skill and yields spots about 150–200 µm in diameter (i.e., 1600 spots/cm²) [54,69]. However, contact-printing robots cannot align their pins to the prefabricated structures and may cause damage to the substrates, especially gel-based surface materials [70].

#### 20.3.3.2.2 Ink-Jet Printing

Ink-jet printers do not contact the printing substrate, thereby avoiding the possible damage caused by the contact [71]. Piezoelectric dispensers are the main type of ink-jet printers used for protein microarrays because they allow the recovery of the portion of the sample that is not dispensed [72] and there is no change in temperature involved in the printing process. These printers are equipped with borosilicate glass capillaries surrounded by a piezoelectric-element collar. The sample is dispensed by the application of a voltage to the piezoelectric collar, typically resulting in the release of a droplet of less than 1 nL [72]. They are generally believed to yield the lowest spot-to-spot variability in the amount of antibodies deposited [73]. However, the ink-jet microarrayer is slow when spotting many different samples and the shearing force during drop formation may cause damage to samples [74].

#### 20.3.3.2.3 DNA-Directed Immobilization

Since DNA patterning has been successfully developed for the fabrication of DNA microarrays, protein chips can be formed on the base of a DNA chip. DNA can be a good chaperone to direct and immobilize protein at the desired spot. By designing the DNA sequence on the microchip and the complementary chaperone DNA tagged to proteins, different proteins can be directed and immobilized to the desired spots by self-assembly. Single-stranded DNA–streptavidin conjugates can be molecular adapters for the tagging of biotinylated proteins with DNA [75–78]. Hence, the setup of
the microscale fluorescence immunoassay is readily configurable from the modular reagents used, i.e., covalent DNA–streptavidin conjugates, biotinylated antibodies, and a microarray containing complementary DNA capture oligomers. As an additional advantage of DDI in immunoassay applications, the binding of the target antigen by antibodies can be carried out in a homogeneous solution, instead of by heterogeneous solid-phase immunosorption, and subsequently the immuno-complexes formed are captured at the DNA microarray by nucleic acid hybridization [79,80]. Wacker et al. [78] have compared DDI with direct spotting of antibodies on chemically activated glass slides and with immobilization of biotinylated antibodies on streptavidin-coated slides. Although all three methods had a LOD of 150 pg/mL for the detection of rabbit IgG, DDI had higher fluorescence intensities than streptavidin–biotin attachment and the best spot homogeneity, as well as experimental reproducibility. Also, a 100-fold less antibody is needed for preparing an array by DDI than by direct spotting. However, DDI is more difficult to perform compared with the other methods.

20.3.3.2.4 Microfluidic Network Patterning

Microfluidic network patterning localizes chemical reactions between the biomolecules and the surface, requiring microliters of reagent to cover square millimeter-sized areas. Immunoglobulins patterned on substrates by μFNs remain strictly confined to areas enclosed by the network with submicron resolution and are viable for subsequent use in assays [80]. This approach to localize chemical reactions on surfaces is based on the definition of open networks of conduits in an elastomeric polymer, poly(dimethylsiloxane) (PDMS) formed by molding the polymer on a lithographically defined master, to form μFNs. When applied to a substrate, the structured elastomer seals the surface by its conformal contact and makes linked, closed capillaries that are filled with liquid reactants and guides them along these conduits with great fidelity to the pattern defined in the elastomer (Figure 20.3) [82]. This patterning method is generic and suggests a practical way to incorporate biological materials on substrates such as gold, glass, or polystyrene. However, it is difficult to use for high-throughput patterning.

FIGURE 20.3 Pattern of biomolecules and sample assays using microfluidic devices: (a) biomolecules are loaded into horizontally oriented channels in the flow chamber module, (b) samples flow through vertically oriented channels in the sample flow chamber module. (Modified from Bernard, A., Michel, B., and Delamarche, E., Anal. Chem., 73, 8, 2001.)
20.3.3.3 Assay Platforms

Most of the microchip-based immunoassays use an ELISA-type platform that consists of incubating and washing steps with shaking. They are slower than flow systems and lack the possibility of simultaneous analysis of different samples on one chip because the entire chip usually can only be exposed to one sample at a time. This does not allow a direct, accurate comparison of different samples without deviations in the results caused by interchip variations, which can range between $12\%$ and $60\%$ depending on the coating of the microarray [55]. However, an accurate comparison between samples and standards is important for quantitative assays, which typically require approximately 10 samples for standard curves, negative controls, and several serial sample dilutions. Some other platforms based on flow systems have been reported to improve the assay performances.

20.3.3.3.1 Microfluidic Network

This platform is based on patterning lines of biomolecules onto a surface by means of a $\mu$FN [83–86]. Solutions to be analyzed are delivered by the multiple channels of a second $\mu$FN across the pattern of antigens using capillary forces [85], thus allowing a direct and accurate comparison of different samples. Individual assays are conducted using independent channels (Figure 20.3). These assays use a sequential series of samples, reagents, and buffers that are displaced one after the other over the PDMS surface, and as these assays are conducted under microfluidic conditions, they are fast, economical in their use of reagents, highly integrated, and yield high-quality signals [84]. After the analysis processing is completed, the flow chamber module can be easily removed to allow for the optical readout. Rowe et al. [86] employed this platform to simultaneously detect staphylococcal enterotoxin B, F1 antigen from Yersinia pestis, and D-dimer in spiked clinical samples in 35 min with LODs of 50, 625, and 500 ng/mL, respectively. Although this platform is fast and sensitive, it is not easy to perform high-throughput assays because the analyte number depends on the number of microfluidic channels.

20.3.3.3.2 Multiple Spotting Technology

Multiple spotting technology (MIST) transfers individual samples by spotting them on top of the different immobilized biomolecules, thereby allowing a multiplex analysis of different samples on a single chip. In the first spotting, biomolecules such as antibodies are transferred to a slide and then the samples and standards, corresponding to the applied antibodies in the first spotting, are transferred to the chip by the second spotting for interaction [87,88]. After binding, the slides are washed and scanned in a microarray scanner. This platform does not apply the samples by total incubations, but it allows the transfer of a multitude of different samples to different spots. Therefore, accurate comparisons and much smaller amounts of samples are possible since the volumes of spotted solution are only 0.19 or 0.6 nL depending on the pins [55]. This technique was able to detect down to 400 zmol of analytes. Moreover, the need for extra incubation time for the binding reaction is eliminated, thereby reducing the assay time. However, this platform relies on the precise alignment of spotting. Since those spots are extremely small (150–200 $\mu$m in diameter), very careful alignment is required to match the spots.

20.3.3.3.3 Filtration-Based Microchips

A filtration-based microchip utilizes a filtration assay with protein microarrays printed on protein-permeable nitrocellulose filter membranes. Instead of incubating and shaking for the binding reaction to occur as with the conventional microarray, the sample is filtered through the micro-array-containing membrane chip to facilitate the binding between analytes and their corresponding capture molecules [89]. Compared with protein microarrays formed on an impermeable solid surface, such as glass slides, this assay platform overcomes the diffusion limitations and enhances the assay sensitivity and specificity [89]. However, this platform does not allow for analysis of multiple samples on the same chip.
20.3.3.4 Detection Systems

20.3.3.4.1 Label Detection
The confocal laser scanner and the charge-coupled device (CCD) imaging detector are the two main detection systems for the analysis of fluorescence-based microchips [73]. The confocal laser scanner provides more sensitive detection than CCD does, but it is slower than CCD since it scans every spot individually on the microchip. Evanescent wave excitation of a planar waveguide has been integrated with a microchip and a CCD camera to identify signals simultaneously across the entire area of the planar waveguide [83, 86, 90]. The evanescent wave, an electromagnetic component of the light guided down the microscope slide, is used for excitation and extends out from the surface of the microscope slide into the lower refractive index medium, decaying exponentially with distance from the surface (Figure 20.4). Because an evanescent field only extends a few hundred nanometers into the solution, only the surface-bound fluorophores are excited, thereby greatly eliminating nonspecific signals [91]. The planar waveguide technology was successfully applied to detect 1 pg/mL of interleukin-6 [92]. Another alternative non-fluorescent label is resonance light-scattering colloidal gold particles that scatter light intensely and quantitative readouts can also be obtained with a CCD imaging system [73]. The LOD could be as low as 1 pg/mL depending on the antibody pair.

20.3.3.4.2 Label-Free Detection
Because labeling molecules sometimes affect protein activity and are restricted to the available detection channels, label-free detection has advantages as a direct detection approach for antibody microarrays. Also, non-label methods have simpler protocols and can provide real-time measurement. SPR, mass spectrometry (MS), SPR-MS, imaging ellipsometry, atomic force microscopy (AFM), and Kelvin nanoprobe have been reported for label-free detection on microchips.

Surface plasmon resonance biosensors use an evanescent field to quantify interactions between analytes and surface-immobilized ligands by changes in surface refractive index, thus providing real-time measurement of biomolecular interactions without labeling and washing steps [66, 67]. The feature of not requiring washing is especially important for low-affinity antibody–antigen interactions that would not be stable if washed before analysis. Samsonova et al. [93] used the Biacore SPR biosensor to measure the antiparasitic agent ivermectin and were able to detect down to 19.1 ng/L in bovine liver. Although SPR is a good detection system, it takes longer than fluorescence-label detection for the analysis of multiple spots.

![Evanescent Wave Excitation in a Planar Waveguide](Image)

**FIGURE 20.4** Evanescent wave excitation in a planar waveguide. The evanescent field only excites the fluorophores on or near the surface. (Modified from Joos, T.O., Stoll, D., and Templin, M.F., *Curr. Opin. Chem. Biol.*, 6, 76, 2002.)
Mass spectrometry can be used to determine the structural features of bound proteins. Surface-enhanced laser desorption/ionization (SELDI)-MS has been used to detect captured proteins in an array on a metal surface. With SELDI, protein arrays act as a surface to which the sample binds uniformly, and the matrix is placed on the microchip after the proteins have been attached. The captured proteins are vaporized using a laser beam, followed by the analysis of the mass spectra to reveal the identities of these proteins [68]. Matrix-assisted laser desorption/ionization coupled to a time-of-flight (MALDI-TOF) mass spectrometer has also been used to sublimate and ionize the samples out of a dry, crystalline matrix via laser pulses for the MS analysis of microchips [94]. A LOD of 20 fmol of antigen/spot was obtained with chips using MALDI-TOF. However, if the matrix material is acidic, the target protein may detach from the surface and thus escape detection [95]. In comparison, the spectra obtained from SELDI are more uniform and reproducible than MALDI-TOF spectra [96].

Because SPR is used for protein quantification as well as real-time interaction analysis, and MS is used to determine the structural features of proteins, the combination of SPR and MS offers unique capabilities for complete protein analysis. Since SPR detection is nondestructive, proteins retrieved from the SPR sensing surface can be further analyzed by MS [97].

Ellipsometry is a nondestructive method for determining film thickness with a resolution of 0.01 nm or better based on the detection of phase shift during reflection of a plane of polarized light [98]. Imaging ellipsometry is an enhancement of standard single-beam ellipsometry that combines the power of ellipsometry with microscopy [99]. High-spatial resolution on the order of micrometers (laterally) and sub-nanometers (vertically) can be achieved in bioaffinity-based sensing by an immunosensor based on imaging ellipsometry. Wang and Jin [98] demonstrated that an immunochip based on imaging ellipsometry was able to not only detect multiple analytes simultaneously without any labeling, but also monitor multiple interaction processes in real-time and in-situ conditions.

The AFM method detects changes in surface topography with a force probe to identify proteins captured in an antibody array. The atomically sharp probe is scanned over a surface with feedback mechanisms that enable the piezoelectric scanners to maintain the probe at a constant force (to obtain height information), or height above the sample surface (to obtain force information). The detection system does not measure force or height directly. It senses the deflection of the cantilever with the probe at its end. Generally, a light beam is reflected from the mirrored surface on the backside of the cantilever onto a position-sensitive photodetector. A small deflection of the cantilever will tilt the reflected beam and change the position of the beam on the photodetector. This approach relies on the change in the height or force that results from ligand-receptor binding, and therefore, does not require the use of labeled receptors. Height changes of 3–4 nm have been observed as a consequence of adsorption of antigenic IgG to a gold or SiO2 surface, followed by an additional increase upon antibody–antigen binding [1,100].

The Kelvin nanoprobe makes use of the principles of Kelvin physics and the AFM. The nanoprobe measures the current generated when two materials, one subjected to vibration, are connected. When contact occurs, the equilibration of the fermi levels of the two substrates leads to a current [101]. The probe uses an AFM-like tip as one of the materials and can detect both topographic and surface-potential maps of a planar surface. It has been used to detect antibody–antigen interactions in a label-free protocol through measurement of the surface potential of the biochemical pair on indium tin oxide, amine-treated slides, and gold substrates [102,103].

Although there are a variety of label-free techniques for protein microarrays, the novelty of the approach or the special expertise and equipment required appear to have limited the use of these non-fluorescent approaches [104].

20.3.3.5 Examples of Microchip Assays

Direct [105], sandwich [73,106], and competitive [67,107] multiplexed immunoassays have all been used in microchips. Below are some examples.
Schweitzer et al. [106] used sandwich immunoassays to measure 75 cytokines on two separate arrays using rolling-circle amplification (RCA). RCA labels antibodies with DNA. Once the detection antibodies are localized to the antigens on the protein array features, their DNA sequences are extended by a DNA polymerase with circular DNA in situ. Therefore, they form long DNA polymers of defined sequence that are tethered to the detection antibody. After this polymerization step, the extended DNA sequence is allowed to hybridize to fluorescently labeled DNA of complementary sequence. Since the extended DNA polymers are very long, multiple DNA labeled with fluorophores are attached to each detection antibody, thereby amplifying the signal. Hence, detection limits as low as 0.5 pg/mL and a dynamic range of 3 decades were achieved. However, the additional procedures for signal amplification increase the assay time and experimental difficulties.

Sreekumar et al. [108] created antibody arrays with 146 distinct antibodies against proteins involved in stress response, cell cycle progression, and apoptosis on poly-L-lysine-coated or superaldehyde-modified glass slides. Microcontact printing was used to monitor the alternations of protein levels in LoVo colon carcinoma cells that were treated with ionizing radiation. The reference standards and samples were labeled separately using either Cyanine 5 or Cyanine 3 dyes. The slides were incubated with a labeled protein mixture and washed with buffer. The signals were detected by a confocal microarray scanner. They observed differential expression profiles with radiation-induced up-regulation of apoptotic regulators such as p53, DNA fragmentation factors, and tumor necrosis factor-related ligands.

Delehanty and Ligler [109] developed an antibody microchip for the rapid detection of proteins and bacterial analytes. A piezoelectric noncontact dispenser was used to immobilize biotinylated capture antibodies on the surface of an avidin-coated glass slide. A microfluidic six-channel flow module was used to conduct the assay by sequentially introducing samples, detection antibodies, and wash buffers. The signals in the microchip were measured by a scanning confocal microscope. Assays were able to be completed in 15 min, and cholera toxin, staphylococcal enterotoxin B, ricin, as well as Bacillus globibii were detected at concentrations as low as 8 ng/mL, 4 ng/mL, 10 ng/mL and 6.2 × 10⁴ CFU/mL, respectively.

Taitt et al. [91] patterned biotinylated antibodies in μFNs (three channels) on avidin-coated glass slides and conducted the assay with microfluidic devices (three channels), forming a 3 × 3 array for nine analytes. Evanscent wave excitation of a planar waveguide was used and a CCD camera was employed for visualization and quantification of the spots. Staphylococcal enterotoxin B, ricin, cholera toxin, Bacillus anthracis Sterne, B. globigii, Francisella tularensis LVS, Y. pestis F1 antigen, MS2 coliphage, and Salmonella typhimurium were detectable at concentrations of 100 ng/mL, 200 ng/mL, 100 ng/mL, 1.5 × 10⁴ CFU/mL, 1 × 10⁵ CFU/mL, 9 × 10⁶ CFU/mL, 100 ng/mL, 1 × 10⁹ PFU/mL and 5 × 10⁶ CFU/mL, respectively.

Knecht et al. [67] reported the simultaneous detection of 10 antibiotics in milk using an automated microarray system. They chose an indirect competitive immunoassay format of immobilizing haptons on glass slides modified with (3-glycidyloxypropyl) trimethoxysilane by a non-contact piezoelectric arrayer. Antibody binding was detected by a second antibody labeled with horseradish peroxidase generating enhanced chemiluminescence, which was recorded with a CCD camera. An analysis was carried out in milk within 5 min since all liquid handling was fully automated. The detection limits ranged from 0.12 to 32 μg/L. The LODs for all antibiotics except penicillin G were far below the maximum residue limits.

### 20.3.4 Microtiter Plate Assays

A typical microtiter plate contains 96 wells, and some even have 384 wells; hence it is easy to immobilize biomolecules in separate wells in an array format by adsorption. Several multiplexed immunoassays using microtiter plates have been reported [110–112]. Another very interesting approach for multiplexed immunoassays using microtiter plates is to have a microarray platform at each well, which can greatly increase the assay throughput (Figure 20.5). A microprinter for
microchips is adapted to print microspots by adsorption in the wells of a regular polystyrene microtiter plate [113], a silanized glass plate [114], or an N-hydroxysuccinimide-activated glass plate [115]. More than 100-spot displays in a single well have been reported [73,115]. A CCD camera is generally used to quantitatively image the arrays. Compared to a regular glass slide-based microarray, this microarray provides high sample throughput, thereby allowing a direct, accurate comparison of different samples without deviation of the results by interchip variations. In addition, it also minimizes nonspecific cross-reactivity between numerous antigen and antibody mixtures, thus maintaining the integrity of the assay [115]. However, it is more difficult to print biomolecules on a microtiter plate than on a glass slide. For combining the advantages of both microtiter plates and glass slides for protein microarrays, Jones et al. [116] fabricated 96 arrays on a glass substrate to match the spacing of a microtiter plate. After printing, the glass substrate was attached to a bottomless 96-well plate using an intervening silicone gasket. Although this technique was used for the study of quantitative protein interaction, it can also be adapted to multiplexed immunoassays.

20.3.5 Bead Assays

Bead-based immunoassays immobilize biomolecules, such as antibodies, onto distinct color-coded polystyrene microspheres embedded with precise ratios of red and infrared fluorescent dyes [117]. Each color-coded bead is identified by a fluorescent signal measured in a flow cytometer. Fluorescently labeled detection antibodies are employed as sandwich immunoassays to quantify the amount of captured targets on each individual bead. The signal intensities from labeled detection antibodies...
are measured by a second fluorescent signal measurement. In general, a flow cytometer is comprised of a red and a green laser, which classifies the beads and quantifies the antigens, respectively (Figure 20.6). Only the fluorophores that are bound to the surface of the beads are counted in the flow cytometer, so it is possible to perform the assay without washing steps. While it has been possible to produce up to 100 distinct beads [118], compared to the thousands of spots on a microchip, bead-based immunoassays still provide a lower throughput.

Rao et al. [118] used the Luminex bead-based system to simultaneously detect bacterial and viral proteins and compared them with microchip-based multiplexed immunoassays. The bead-based system showed lower LODs of 7.05 x 10^4 CFU/mL and 3.51 x 10^6 PFU/mL for the detection of B. globigii and the RNA bacteriophage virus (MS2), respectively, while microchips were more amenable to miniaturization. McBride et al. [117] also used the same system to demonstrate the simultaneous detection of four simulants of biological warfare agents, comprising a virus (MS2), protein toxins (ovalbumin), bacterial spores (B. globigii spores), and vegetative cells (Erwinia herbicola) in 1 h with LODs of 4.2 x 10^7 PFU/mL, 1 μg/L, 1.5 x 10^4 CFU/mL, and 5 x 10^4 CFU/mL, respectively.

**20.3.6 Electrode Assays**

Although most multiplexed immunoassays are based on optical biosensors, amperometric multiplexed immunoassays have been developed as well [119–121]. Different antibodies are immobilized on a variety of working electrodes [120,121] or on the surface next to the working electrodes [119] by adsorption [119], photolithography [120], or DDI [121]. Horseradish peroxidase or alkaline phosphatase was labeled to the detection antibody. An electric current was produced, and
thus detected by the electrodes, after the enzyme reacted with the substrate. Simultaneous detection of five analytes (α1 acid glycoprotein, ricin, M13 phage, B. globigii spores, and fluorescein) based on the redox enzyme (horseradish peroxidase) amplified electrochemical detection was demonstrated recently by Dill et al. using microelectrode arrays [121]. Horseradish peroxidase catalyzed the oxidation of a substrate, while using peroxide as the electron acceptor, yielding a high-rate enzyme turnover. The LODs were in the attomole range and the dynamic range was 4–5 decades of analyte concentration with an assay volume of 50 μL or less. Because complementary metal oxide semiconductor (CMOS)-based silicon chip was employed, the microelectrode arrays could provide over 1000 electrodes/cm². However, it seems that only DDI is suitable for use on microelectrodes among the entire antibody patterning methods mentioned above.

20.3.7 NanoWire Assays
Another kind of electrical multiplexed biosensors are silicon-nanowire field-effect devices [122,123]. Unlike most electrode assays, it is label-free, real time, and antibodies can be spotted on the nanowire surfaces by microchip arrayers. Analytes will cause sensitive changes in conductance when they bind to antibodies on the nanowire surfaces. Zheng et al. [123] have demonstrated the multiplexed detection of cancer markers, prostate-specific antigen (PSA), PSA-α1-antichymotrypsin, carcinoembryonic antigen, and mucin-1 using the nanowire sensor chip. Protein-sensing experiments were performed in microfluidic channels formed by a PDMS polymer sealed to the chip. Concentrations as low as 0.9 pg/mL of PSA can be detected in undiluted serum samples. The authors also compared their work to SPR detection of marker proteins [124–126] which showed the LOD of 10–100 pg/mL.

Although nanowire arrays can perform a sensitive, label-free, and real-time detection, the fabrication was complicated, including photolithography and metal deposition steps [123]. Also, the simultaneous real-time measurements were limited to three distinct nanowire sensor devices because of the availability of measurement electronics. Although at least 100 independently addressable sensor elements were available in the arrays and could be used with more sophisticated multiplexing electronics [123], it was still relatively low throughput, compared to thousands of spots on protein microarrays.

20.4 Multiplexed Immunoassay Challenges
Although there are many monoclonal and polyclonal antibodies commercially available, most are very expensive and many of them do not have high enough affinity and specificity for multiplexed immunoassays. To replace expensive, low-affinity, or low-specificity antibody, recombinant antibodies have been made by phage display [127,128], ribosomal display [129,130], or yeast display [131,132]. Different antibodies display widely varying performance in immunoassays attributed to varying affinities [111,133]. Phage display of antibody fragments is the most popular method for generating recombinant antibodies and has been successfully used to increase antibody affinity more than 1000-fold [134]. Another great advantage of recombinant antibodies is the relatively small molecular weight, thereby facilitating high-density immobilization on a support surface. Also, antibody engineering for the oriented attachment on the solid surface is also possible [135]. In addition to the challenge of antibody resources, the assay performance is also a big challenge. Different antibodies have different optimum performance conditions such as pH, ionic strength, temperature, and time. Sometimes, it is necessary to sacrifice individual antibody performance to achieve the overall performance of multiplexed immunoassays.

The labeling of antibodies is also a challenge for multiplexed immunoassays, especially on a massively parallel scale. The strategies for conjugating labels to antibodies mostly involve covalent binding, which is usually complicated and time consuming [136–139]. In addition, these methods generally require the use of the amino groups on the antibody. Even the non-covalent biotin-(strept)
avidin coupling method also involves the biotinylation of antibodies [140]. These approaches, however, are limited because most antibodies contain randomly distributed amino groups, leading to multiple attachment sites. The random nature of this attachment can cause some of the conjugated antibodies to lose antigen-binding activity because of direct chemical modification or steric hindrance of the antigen-binding site [141,142].

To have a simple labeling procedure and improve the affinity of labeled antibodies, IgG Fc-binding proteins, like protein A or protein G, can be used to couple to the antibodies. A genetically fused protein A-luciferase has been developed for bioluminescent immunoassays [143]. Protein G also binds to the IgG Fc fragment, and it represents a more general and versatile IgG-binding reagent [144]. It binds a wider range of IgG subclasses and a greater variety of mammalian species with higher affinity than protein A. For example, protein G has strong affinity to goat IgG, but protein A barely binds to goat IgG. In addition, protein G is not as pH dependent as protein A when binding to immunoglobulins [144–146]. Protein G-based universal reagents have been developed for time-resolved immunofluorometry [147]. Up to eight europium atoms were tagged on each protein G, but this greatly reduced the IgG-binding ability of protein G. Another universal reagent for immunoassays was recently developed: protein G-liposomal nanovesicles that were generated by coupling a large amount of protein G to each liposome, thereby not only providing great amplification but also retaining the IgG-binding ability of protein G (decreased by only 5.3%) [111,148]. Liposomal nanovesicles, i.e., liposomes, are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous cavity. Because each liposomal nanovesicle can contain up to several million fluorescent dye molecules, thereby providing greatly enhanced signals, liposomal nanovesicles have been successfully used as reporter particles in immunoassays [149–152]. The process of antibody coupling to protein G-liposomal nanovesicles is simple and rapid (30 min or less) without reducing the affinity of the labeled antibody. A universal reagent like protein G-liposomal nanovesicles is desirable for facile labeling of a large number of different antibodies for multiplexed immunoassays.

20.5 CONCLUSIONS

Only a few multiple-label immunoassays, such as multiple lanthanides and multiple nanocrystals, can simultaneously detect more than three analytes because of the difficulty of discriminating a larger number of distinct simultaneously detectable labels. Spatially resolved immunoassays, however, can easily provide higher throughput, especially in miniaturized multiplexed immunoassays. Because of advances in micro/nanotechnology, antibody microarrays using microchips, beads, microtiter plates, microelectrodes, and nanowires can operate in a high-throughput mode to detect more than 100 analytes simultaneously. However, only microchip-based immunoassays have really demonstrated high throughput. Continued research in nanotechnology is expected to be the key to increasing the throughput and reducing the assay time and cost. Recombinant antibody technology will also play a major role in improving the sensitivity and specificity of the simultaneous multianalyte immunoassays. Thus, future developments in the promising field of simultaneous multianalyte immunoassays are linked to the continued progress in nanotechnology and recombinant antibody technology.

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REFERENCES


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**AUTHOR QUERIES**

[AQ1] Please check if the change made in the unit in the sentence “Samsonova et al. used... liver” is OK.

[AQ2] The text citation of Reference 81 is missing. Please check and provide.

[AQ3] Please update Reference 111.

[AQ4] Please provide the volume number and issue number in Reference 116, if appropriate.