ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

Volume 1 Issue 1 2007

www.abap.in

Current Trends in Biotechnology and Pharmacy
ISSN 0973-8916

(Electronic Version)

www.abap.in

Current Trends in Biotechnology and Pharmacy

ISSN 0973-8916

Volume.1 (1) **October - 2007**

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Current Trends in Biotechnology and Pharmacy, Vol.1 (1) 1-21 (2007)
ISSN:0973 - 8916

Recent Developments in Multianalyte Immunoassay

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Abstract

Multianalyte immunoassay has gained increasing attention due to its high sample throughput, short assay time, low sample consumption and reduced overall cost per assay. Most of the current developed approaches for multianalyte immunoassay are based on spatial-resolved, multilabel or separation mode. This paper reviews the progress of multianalyte immunoassay and its applications in different fields with 90 references. The outlook of this promising technique has been discussed.

Keywords

Immunoassay; Immunosensor; Multianalyte immunoassay; Array; Review

Introduction

As a promising approach for selective and sensitive analysis, immunoassay has recently gained increasing attention in different fields including environmental monitoring, clinical diagnosis, food safety, pharmaceutical analysis and bacteria identification. It is often necessary to monitor or quantitate several components in a complex system. For example, due to the limited specificity and sensitivity of biomarkers for clinical diagnosis, the measurement of a single biomarker is usually insufficient for diagnostic purpose. Some studies have showed that the measurement of biomarkers panel can avoid false positive or false negative results to improve their diagnostic value (1). Traditionally, immunoassay of analytes panel is performed as discrete tests, i.e., one analyte per assay run, and several runs are needed to detect all components in a complex system. Great consumptions of time, reagent and labor limit the application. To dissolve these limitations, multianalyte immunoassay (MAIA) that can measure two or more analytes in a single run has become a long-cherished goal of immunochemist since simultaneous radioimmunoassay of human insulin and growth hormone in serum sample using I-131 and I-125 as labels was reported in 1966 (2). Compared with parallel single-analyte immunoassay methods, MAIA offers some remarkable advantages, such as high sample throughput, improved assay efficiency, low sample consumption and reduced overall cost per assay (3). This review focuses on the progress and applications of MAIA, including spatial-resolved, multilabel and separation modes.

1. Spatial-Resolved Mode

The spatial resolution of different immunoreaction areas using a universal label for fluorescent, chemiluminescent (CL), spectrophotometric, electrochemical, and piezoelectric detections with array detectors including charge-coupled device (CCD) camera and multichannel electrochemical workstation is the most popular MAIA method.

1.1 Optical detection

Antigen and antibody arrays dotted on planar supports, such as multi-well plate, nylon membrane and glass slide, combined with fluorescent probes (4-10) and enzymes (9, 11-13) as labels are traditionally adopted to perform spatial-resolved MAIA using CCD and laser scanner detector.

Weller's group (14) proposed a parallel affinity sensor array for the rapid analysis of 10 antibiotics in milk. Microscope glass slide modified with (3-glycidyloxypropyl) trimethoxysilane was used for the preparation of hapten microarray and inserted into a flow cell to act as an automated flow-through CL multianalyte immunosensor. After incubation process, the horseradish peroxidase labeled immunocomplexes of the 10 antibiotics generated enhanced CL signals, which were recorded with a CCD camera. The fully automated liquid handling and sample processing enabled one analysis cycle to be completed in less than 5 min. With the similar device and protocol, multiple herbicides (15, 16) and allergen-specific IgE in human serum (17) have been assayed in array mode.

Jiang et al. (18) reported a miniaturized, microfluidic version of serial-dilution fluorescent immunoassay for antibodies in HIV+ human serum. In this assay, serially diluted solutions of serum flowed in channels across orthogonal, parallel strips of HIV ENV proteins (gp41 and pg 120) adsorbed on a polycarbonate membrane. The bound antibodies could be measured using a second, fluorescent labeled antibody. This assay used a microdilutor network to achieve serial dilution and allowed simultaneous, quantitative analysis of multiple analytes with high concentrations on a single chip.

Some immunosensors arrays composed of recognition component, fluidics component for movement of various solutions and detector for collection of signals produced from positive samples have been developed at the Navel Research Laboratory, USA. Sandwich fluoroimmunoassays are performed on the surface of microscope slides previously patterned with stripes of capture antibodies. After both sample and fluorescent tracer antibodies are introduced in a direction perpendicular patterned with stripes of capture antibodies, the immunocomplexes formed can be observed as a checkerboard pattern of fluorescent spots excited by evanescent wave on the surface. These array immunosensors have been successfully applied in MAIA of proteins, bacterias and biohazards (19-27).

Barzen et al. (28-30) proposed several optical multianalyte immunosensors for environment pollutants based on flow-injection immunoassay coupled with total internal reflection fluorescent detection. They immobilized haptens on the different areas of transducer surface of the flow cell and determined simultaneously multiple pollutants in a spatial-resolved and competitive mode. Rodriguez-Mozaz et al. (31) simultaneously detected atrazine, isoproturon and estrogen estrone in river water using an immunosensor fabricated with a similar protocol (Figure 1). The performance of the developed immunosensor was evaluated against a well-accepted traditional method based on solid-phase extraction followed by liquid chromatography-mass spectrometry, and the results obtained from the two methods indicated good agreement.

A one-step lateral flow immunoassay on a strip format for the rapid and simultaneous detection of free and total prostate specific antigens (f-PSA and t-PSA) and estimation of f-PSA to t-PSA ratio (f/t-PSA) in serum has been reported (32). Herein, f-PSA or t-PSA is sandwiched between anti-f-PSA or anti-t-PSA monoclonal antibodies parallel immobilized on the strip and a colloidal gold labeled anti-PSA tracer antibody. The

presence of f-PSA and t-PSA results in the appearance of two parallel pink colour lines. Two membrane-based competitive immunoassays using gold particles and horseradish peroxidase (HRP) as tracers in lateral flow format have also been developed for MAIAs of carbaryl and endosulfan (33). The visual detection limits for carbaryl and endosulfan are 100 and 10 μg/L with gold and 10 and 1 μg/L with HRP as labels, respectively.

Yacoub-George et al. (34) designed a portable multichannel immunosensor for biological warfare agents, which was based on a capillary ELISA technique in combination with a miniaturized fluidics system and used CL as the detection principle (Figure 2). The fluidic system allowed three CL immunoassays to be performed simultaneously within three fused silica capillaries with three photodiodes as detectors. Koch et al. (35) also presented a portable optical multichannel immunosensor for the simultaneous operation of three flow-through capillary enzyme immunoassays. The parallel operation was achieved by stop-flow incubation. When one capillary was in the process of signal collection, the other two were in incubation procedure. This work represented a versatile tool for immunoassay of several biological warfare agents in parallel with only one non-array detector.

The application of a surface plasmon resonance-based biosensor with four flow channels in combination with a mixture of four specific antibodies resulted in a competitive inhibition MAIA for the simultaneous detection of five aminoglycosides in reconstituted skimmed milk (36). Chung et al. (37) developed a sequential method for the analysis of HRP and bovine serum albumin using a surface plasmon resonance biosensor. Non-array fluorescent detector has also been used for spatial-resolved detection of multiple pesticides (38), hormones (39) and proteins (40) by moving the antigens or antibodies immobilized affinity microcolumn and capillary immunosensor with a motorized translational stage. Owing to the relatively complicated detection device, this strategy needs to be further improved.

1.2 Electrochemical detection

Amperometric immunosensor array fabricated with multiple working electrodes sharing one common counter electrode and reference electrode has been successfully used for MAIA of pepsinogens (41), tumor markers (42-45) and hormones (46). CombiMatrix Corporation (47) developed a microarray of individually addressable electrodes using conventional CMOS integrated circuitry. This microarray system provided a host for MAIA due to the large number of electrodes available, which integrated over 1000 electrodes per square centimeter. The results for human α 1 acid glycoprotein, ricin, M13 phage, *Bacillus globigii* spore, and fluorescein indicated that this method was one of the most sensitive available, with limits of detection in the attomole range. Electrochemical sensor array often suffers from cross-talk due to the diffusion of electroactive product generated at one electrode to a neighboring electrode (43,45). Thus, an enough spatial distance between adjacent electrodes is necessary to cumber the diffusion procedure. Use of double siloxane layer (45) and iridium oxide (42-44) matrix can retard the diffusion of enzyme-generated product to lower cross-talk.

Ju et al. (48,49) proposed two disposable immunosensor arrays for simultaneous electrochemical determination of multiple tumor markers. The low-cost immunosensor arrays were fabricated simply using cellulose acetate membrane to co-immobilize thionine as a mediator and antigens on different working electrodes of a screen-printed chip, on which the immobilized thionine shuttled electrons between HRP labeled to antibodies and the electrodes for

enzymatic reduction of H_2O_2 to produce detectable signals. This chip could avoid the electrochemical and electronic cross-talks between the electrodes, which enabled the arrays to be miniaturized without considering the distance between immunosensors.

Kong et al. (50,51) proposed two arrays of eight electrodes for label-free capacitive and conductive immunoassay of liver fibrosis markers using ultrathin α 1 alumina sol-gel films and electrochemically deposited polypyrrole to immobilize antibodies, respectively.

1.3. Mass-sensitive detection

Luo et al. (52) constructed a 2x5 model piezoelectric immunosensor array fabricated with disposable quartz crystals for quantification of microalbumin, 1-microglobulin, α 2 microglobulin, and IgG in urine. With the piezoelectric immunosensor array, 4 urinary proteins could be quantified within 15 min. This method had an analytical interval of 0.01-60 mg/L. Similarly, a novel simultaneous immunoassay technique has been developed for the determination of complement factors (C_4,C_5,C_{1q} and B factor) by constructing a piezoelectric quartz crystal array system (53). These mass-sensitive piezoelectric immunosensor arrays can provide a convenient label-free approach to MAIA.

1.4. Optical encoding and addressing

Spatial-resolved arrays are typically manufactured by labor-intensive methods requiring high precision such as ink-jet printing, micromachining, photolithography, and photodeposition. Randomly ordered addressable sensor array developed in Walt's laboratory (54) provided an alternative approach to array fabrication. In this approach, micrometer-sized sensors were produced by immobilizing different recognition molecules on the surface of microparticles encoded using two fluorescent dyes. The addressing procedure was performed by taking the fluorescence intensity at each emission wavelength and then dividing the two values to get the signature of that particular ratio. With this principle, multiple drugs (55), proteins (56-58), biological warfare agents (59) and cytokines (60) were simultaneously detected with CL or fluorescent method and randomly ordered antibodies immobilized copolymer microspheres or metallic particles as microsensors.

Theoretically, thousands of antigens or antibodies can be spotted onto one single planar support to screen thousands of analytes. Although this mode can screen large numbers of analytes, accurate quantitative data in these arrays are usually limited or difficult to be obtained (44). Requiring of complicated and expensive spotting technique with high precision also greatly limits its application. Although optical encoding and addressing allows randomly ordered sensor arrays to be identified for MAIA, the encoding process complicates the manipulation. Furthermore, spatial-resolved MAIA is typically performed with expensive array detector such as CCD camera for optical detection or multi-channel workstation for electrochemical measurement (61).

2. Multilabel Mode

The second dominant mode for MAIA is performed using different labels to tag antibodies or antigens (one per analyte), including radioisotopes, enzymes, fluorescent and metal compounds. Different analytes can be easily distinguished using these labels by such parameters as potential, wavelength, decay time and so on.

2.1 Wavelength resolution

ELISA for MAIA involves labeling the analytes with various enzymes, whose catalyzed reactions can easily be distinguished from each other by

absorption spectra (62, 63). Selection of the enzyme labels is a key step in the development of an ELISA based MAIA. Blake et al. (62) mentioned that the ideal enzyme labels for MAIA should meet the following requirements: (i) the enzymes should be readily available, inexpensive, and have high turnover numbers; (ii) each enzyme must be stable under the selected simultaneous assay conditions and not easily to be interfered by other enzyme or its substrate; (iii) all enzymes must have similar optimal assay conditions; (iv) the assay method for each enzyme should be simple, sensitive, rapid, and cheap; (v) all enzymes should not occur in the practical sample to be assayed, and interfering factors should be absent; (vi) each enzyme should contain potentially reactive groups that allow linking to antigen or antibody while retaining the enzyme activity; (vii) the spectra of the products of the enzyme-catalyzed reactions should not overlap with each other.

Ihara et al. (64) immobilized a mixture of antigenic peptides of FAK and c-Myc to nanospheres with red emission, and a mixture of c-Myc and α catenin to green nanospheres, respectively. As seen in Figure 4 (64), anti-FAK and anti α catenin antibodies could form aggregates with red and green emissions, respectively. The anti-c-Myc antibody could form aggregate emitting yellow light as a result of color overlapping. This strategy enabled specific antibodies to be detected in one-step procedure with color-encoded nanospheres. Swartzman et al. (65) proposed a bead-based two-color MAIA for cytokines IL-6 and IL-8 using Cy5.5 and Cy 5 as fluorescent labels, respectively. The linear dynamic ranges of them were 125-4000 pg/mL and 15.6-2000 pg/mL, respectively. This work utilized fluorometric microvolume assay technology to image and measure bead-bound fluorescence while the background fluorescence was ignored. Consequently, no wash steps were required to remove unbound antibody, ligand, and fluorophore. Goldman et al. (66) used antibody-conjugated quantum dots with emission maximums at 510, 555, 590, and 610 nm to demonstrate multiplex assays for four protein toxins present in the same sample. However, a deconvolution of composite spectra was needed to distinguish the overlapping signals.

2.2. Time resolution

The fluorescence of lanthanide chelates has the advantages of high quantum yield, long decay time, exceptionally large Stoke's shift, and narrow emission peak. Specific chelate fluorescence can be easily distinguished from the sample matrix fluorescence and the scattered light, and the fluorescence from different lanthanides can also be easily discriminated due to their difference in decay time and emission wavelength, which makes the lanthanide chelates preferable to any other probes for developing multilabel-based time-resolved MAIA. Of the 15 lanthanide ions, Eu^{3+}, Sm^{3+}, and Tb^{3+} are the most commonly employed probes, and have been widely used for time-resolved fluorescent MAIA of multiple tumor markers (67), hormones (68), recombinant proteins (69) and antibodies (70).

Ito et al. (71) developed a simple and rapid time-resolved fluoroimmunoassay for simultaneous determination of alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG) and estriol (E3) using Eu^{3+} and Sm^{3+} chelates. In this proposed method, a 96-well microtiter plate for AFP and hCG assay and a transferable solid phase plate for E3 assay were combined to perform MAIA of the three analytes with only two probes. The measurable ranges for AFP, hCG and E3 were 3.91-1000 ng/mL, 877-250 000 IU/L and 0.39-100 ng/mL, respectively.

2.3. Potential resolution

The dual-analyte homogeneous immunoassay of phenobarbital and phenytoin was carried out simultaneously at physiological pH by square-

wave voltammetry on Nafion-loaded carbon paste electrode. Phenobarbital and phenytoin were labeled by cobaltocenium salt and ferroceneammonium salt with standard redox potentials of -1.05V and 0.26 V, respectively. Detection limits of 0.25 and 0.2 mu were achieved for the two antiepileptic drugs, respectively (72). As seen in Figure 5 (73), an electrochemical stripping immunoassay protocol using different inorganic nanocrystal as tracers and magnetic beads as support has been developed for the simultaneous measurements of proteins. Each biorecognition event yields a distinct voltammetric peak, whose position and size reflect the identity and concentration of the corresponding analyte, respectively. This protocol has been used for a simultaneous immunoassay of β 2 microglobulin, IgG, bovine serum albumin, and C-reactive protein using ZnS, CdS, PbS, and CuS colloidal crystals as labels, respectively (73). Hayes et al. (74) proposed a MAIA method for human serum albumin and IgG. Bismuth and indium ions were coupled to the two proteins through the bifunctional chelating agent diethylenetriamine-pentaacetic acid. Following the competitive reactions between unlabeled and labeled proteins for limited amount of specific antibodies immobilized on polystyrene, the bound metal ion labels were released by acidification and detected by differential pulse anodic stripping voltammetry with detection limits of 1.8 and 0.6 pg/mL for human serum albumin and IgG, respectively.

2.4. M/e resolution

Zhang et al. (75) developed a dual-label immunoassay method for the simultaneous determination of AFP and free hCG β in human serum. Monoclonal antibodies immobilized on microtiter plates captured AFP and hCG beta, which were detected by Eu^{3+}-labeled AFP and Sm^{3+}-labeled hCG β tracer antibodies with inductively coupled plasma mass spectrometry (ICPMS) after Eu^{3+} and Sm^{3+} were dissociated from the plates with HNO_3 solution. However, this technique could not be used for microarray detection since it was necessary to dissolve the elemental tags before introducing them into the plasma source. They (76) also reported the detection of multiple proteins on each spot of the immuno-microarray by laser ablation ICPMS. AFP, carcinoembryonic antigen (CEA) and human IgG were detected as model proteins in sandwich format on a microarray with Sm^{3+}-labeled AFP antibody, Eu^{3+}-labeled CEA antibody, and Au nanoparticle-labeled IgG antibody as tracer antibodies. The detection limits were 0.20, 0.14, and 0.012 ng/mL for AFP, CEA, and human IgG, respectively. This detection method allowed detection of multiple analytes from each spot of microarray with a spatial resolution at micrometer range, which could alleviate the stress to fabricate high-density arrays.

2.5. Scintillation energy resolution

In 1966, as the founder of MAIA, Morgan (2) proposed an original simultaneous radioimmunoassay of human insulin and growth hormone in serum sample using I-131 and I-125 as labels and exploiting the difference in scintillation energy produced from the two radioisotopes to discriminate the two analyte. Similarly, the simultaneous radioassay of vitamins (77) and hormones (78) could be carried out using Co-57 and I-125 as probes. Recently, few attention is paid to the radioimmunoassay based MAIA due to the damage of radioisotopes to environment and operator.

2.6. Substrate zone resolution

It has been noted that the different labels used in multilabel mode often need markedly different optimal assay conditions, and traditionally simple combination of multiple labels often leads to loss of assay performance (2). Furthermore,

this mode sometimes suffers from signal overlapping of different labels (66).

Ju et al. (61) designed a substrate zone-resolved multianalyte immunosensing system, with which HRP labeled carcinoma antigen 125 (CA 125) immunocomplex and alkaline phosphatase labeled CEA immunocomplex were sequentially detected in their corresponding CL substrate zones. This designed technique solved two key problems in multilabel mode: one was to obtain distinguishable CL signals without consideration of wavelength, and the other was to enable each CL reaction to be catalyzed by the label in its optimal assay condition without loss of assay performance. Unfortunately, as other MAIA methods based on multilabel mode, this technique encountered a difficulty to find more available enzyme labels, which limited the number of analytes. In order to overcome this limitation, this group further proposed a two-dimensional resolution system of channels and substrate zones (79). Using CA 125, CA 153, CA 199 and CEA as two couples of model analytes, two couples of capture antibodies were immobilized in two channels, respectively. With a sandwich format the CL substrates for alkaline phosphatase and horseradish peroxidase were delivered into the channels sequentially to perform multiplex immunoassay after the sample and trace antibodies were introduced into the channels for on-line incubation. When three or four channels were used in the flow-through device, the detectable analytes in a single run could be 6 or 8, respectively, with a 10 s longer analytical time for each added channel.

3. Separation Mode

Another method coupled with separation techniques such as capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) can be used for MAIA. Competitive immunoassay combined with fluorescent detection is generally adopted to perform CE based MAIA, the analytes includes abused drugs (79, 81) and peptides (82). Obviously this strategy often suffers from the adsorption of immunoreagents on inner wall of the capillary, which can be prevented by optimization of separation buffer type and pH that allowes application of high electric field (82). An on-line coupling of a label-free reflectometric interference spectroscopic biosensor to a HPLC system has been described for MAIA of four pesticides (83). In this system a highly cross-reactive antibody against the four pesticides is used to bind the pesticides. The eluate of the HPLC is mixed continuously with the antibodies, and the presence of antigens is detected by a reduction of the antibody binding to the transducer.

Roda et al. (84) proposed a field-flow fractionation (FFF)-CL based solid-phase competitive immunoassay, in which micrometer-sized beads coated with the capture antibody were used as solid phase, and analyte-HRP conjugate was used as tracer. Once the competitive immunoreaction took place within the injection loop of the system, the antibody-bound tracer was separated from tracer in solution in a few minutes by means of FFF. FFF-based MAIA could be developed by use of beads with different sizes (1-50 mu), each coated with the specific antibody for one analyte. The beads could be fractionated by FFF before CL signals collection to realize detection of multiple analytes in a single run.

The thermosensitive poly (N-isopropylacrylamide) (PNIP) and magnetic beads have been widely utilized as the separation carriers for immunoassays. A fast homogeneous immunoreaction as well as a simple heterogeneous separation process is carried out for MAIA in the light of some certain characteristics of water-soluble PNIP and magnetic beads, and thus, lower nonspecific affinity and higher sensitivity are accomplished

(85). The results of CL detection of IgG and IgA indicate the detection limits as low as 2.0 and 1.5 ng/mL, respectively.

4. Cross-Reactivity

Cross-reactivity is a crucial analytical parameter regarding specificity and reliability of MAIA, which is frequently encountered in MAIA of small molecule analytes. In many cases, the antibodies recognize a variety of analogs and metabolites of the target analyte, for example, some *s*-triazines and their metabolites with similar structures shown in Figure 6 (86). Even monoclonal antibodies are often unable to discriminate absolutely molecular analogs with small structural differences. Efforts to derive monoclonal antibodies to small analytes generally produce panels of antibodies that differ in their cross-reactivity for the primary target analyte and its analogs and metabolites. Antibodies arrays combined with some chemometric means inclusive of neural network are often used to overcome the difficulty in exact quantitation resulted from the cross-reactivity (86-90).

5. Conclusion and Outlook

In recent yeas, MAIA has attracted considerable interest due to its outstanding advantage in assay speed, cost and labor consuming. So far the spatial-resolved mode has been the most popular mode due to its high analyte throughput and large information amount. The further work needs to develop arrays with higher density and simpler preparation protocol using cheaper array detector. Most of the multilabel mode based methods focus on using of lanthanide chelates as labels and time-resolved fluorescent detection. More labels with higher signal resolution degree and less requirement to assay condition are urgently needed. Various resolution methods in time, space, substrates, reactants, labels and detection methods will be designed and developed for MAIA in the future. Military application and environment monitor are anxious to miniaturized, integrated and portable MAIA system fit for field application. MAIA system with high sample throughput and rapid assay speed has great application potential in disease screen.

Acknowledgements

This research was financially supported by the National Science Funds for Distinguished Young Scholars (20325518) and Creative Research Groups (20521503), the Key Program (20535010) from the National Natural Science Foundation of China and the Science Foundation of Jiangsu (BS2006006, BS2006074).

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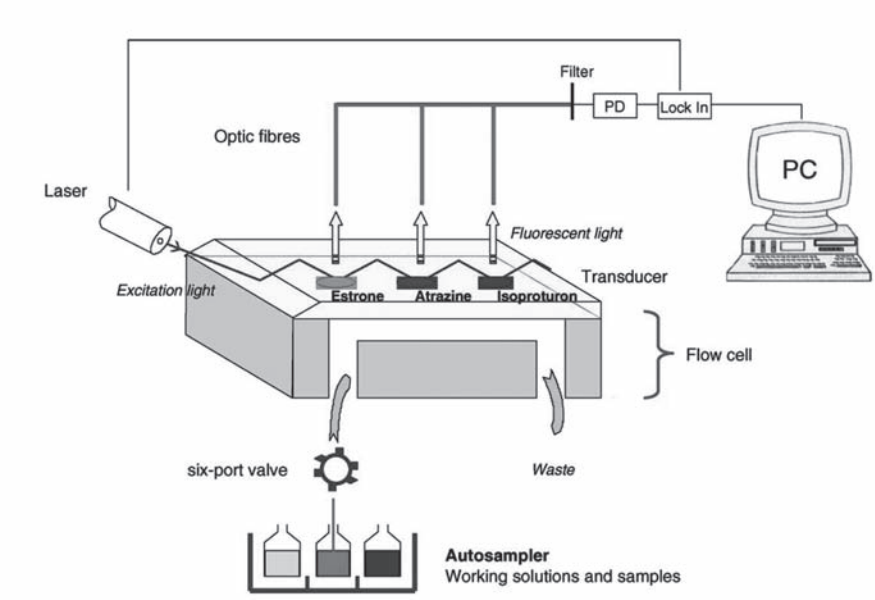

Fig 1 : Scheme of flow-injection immunosensor used for detection of multiple pollutants in river water.

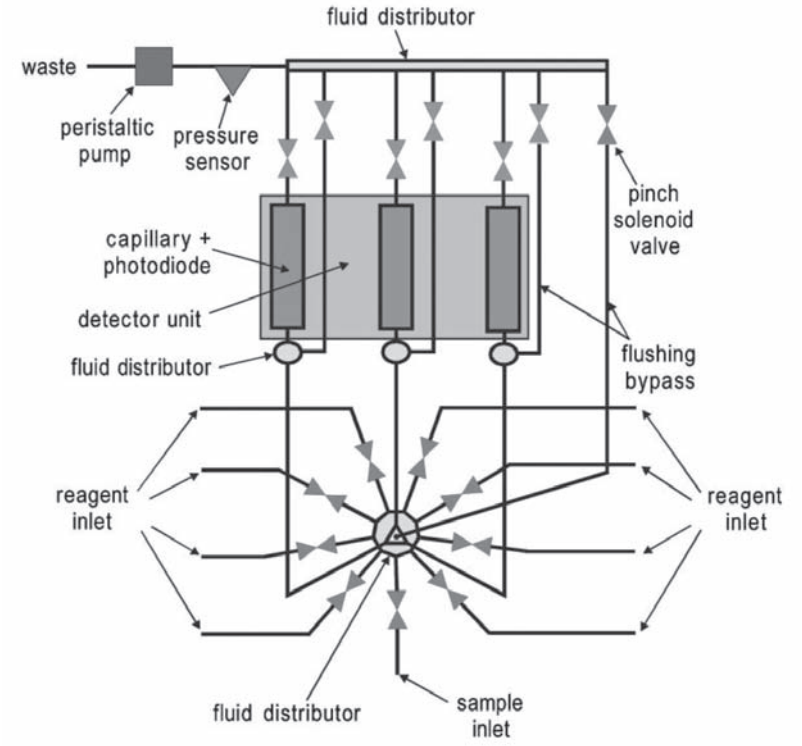

Fig 2 : Scheme representing the arrangement of the fluidics components of the CL multichannel immunosensor for biological warfare agents.

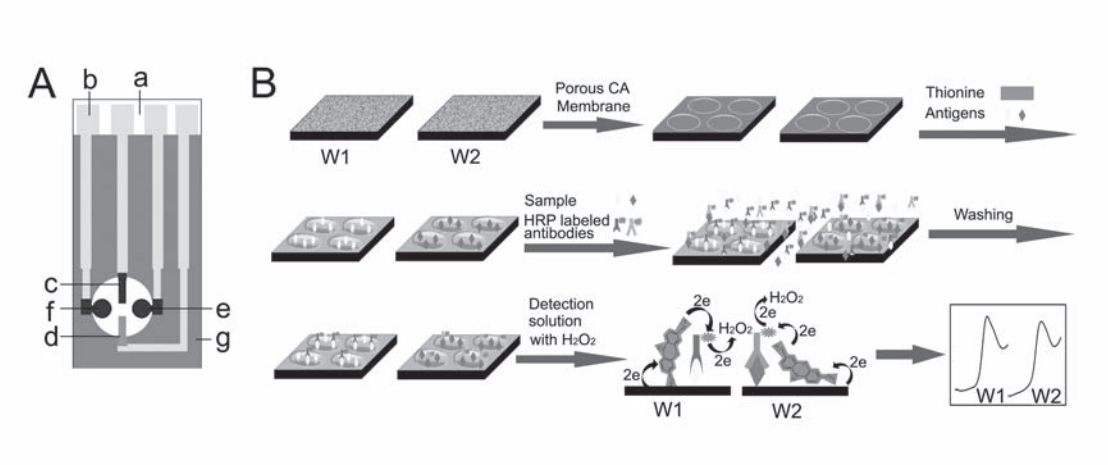

Fig 3 : Schematic diagrams of (A) screen-printed four-electrode system and (B) preparation of immunosensor array and MAIA procedure: (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) Ag/AgCl reference electrode, (e) W1, (f) W2 and (g) insulating dielectric.

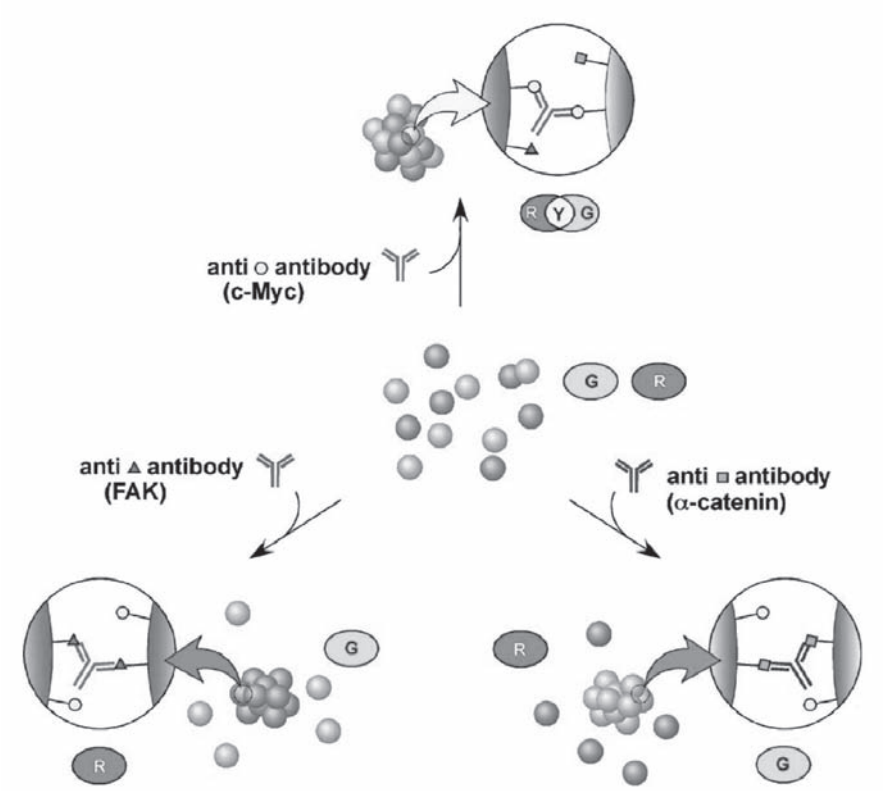

Fig 4 : Schematic illustration of the MAIA using selective aggregation of antigenic peptide-modified nanospheres.

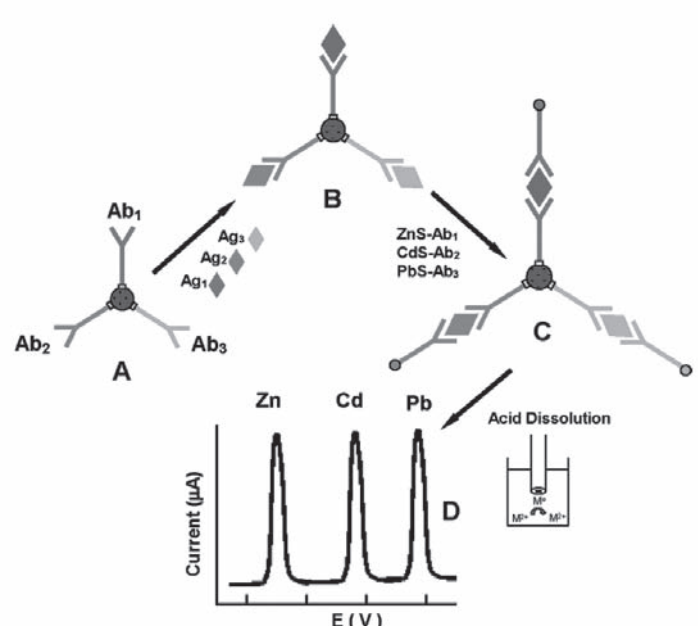

Fig 5 : Multiprotein electrochemical stripping immunoassay protocol using different inorganic nanocrystal tracers: (A) introduction of antibody-immobilized magnetic beads, (B) capture of the antigens to the antibodies-immobilized magnetic beads, (C) capture of the nanocrystal-labeled secondary antibodies and formation of sandwich immunocomplexes, (D) dissolution of nanocrystals and electrochemical stripping detection.

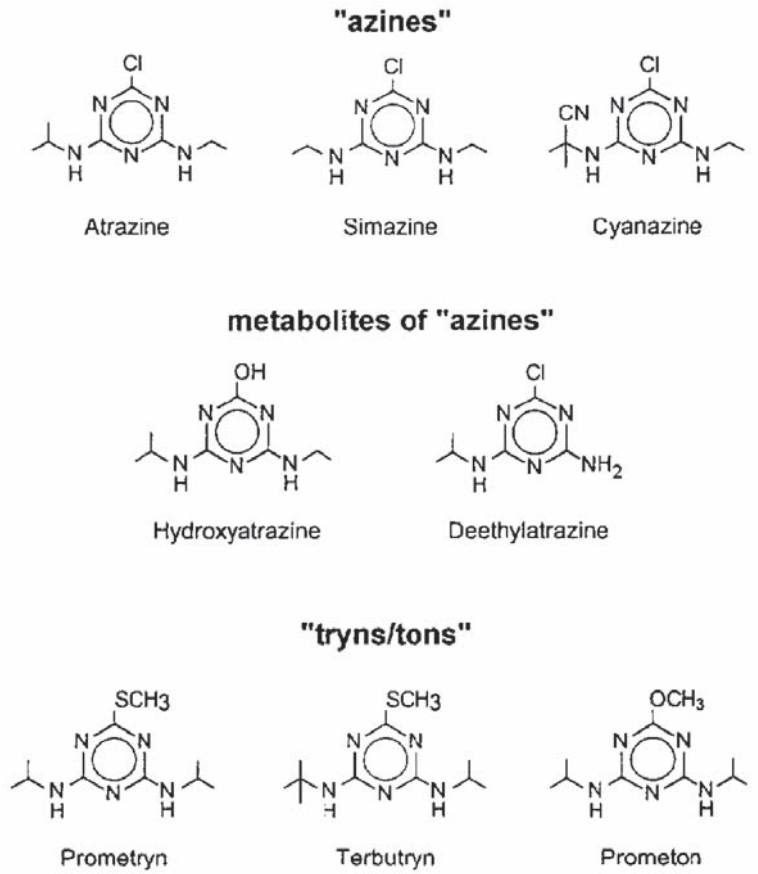

Fig 6 : Some *s*-triazines and their metabolites with similar structures.