

Biomedical and clinical applications of immunoassays and immunosensors for tumor markers

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Cancer is one of the most threatening diseases for human beings. The detection of tumor biomarkers, including serum tumor markers and potential prognostic factors for tumors, plays an important role in clinical diagnoses and evaluation of treatment for patients with certain tumor-associated diseases. Many immunoassay methods and immunosensors are being developed for detection of tumor-related biomarkers. This review summarizes the state of immunoassay technologies for tumor diagnostics. It also focuses on a growing number of applications and progress in immunosensors for tumor markers. It covers the basic principles and biomedical and clinical applications of immunosensors, and indicates the future prospects in this field.

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1. Introduction

Millions of people throughout the world face the risk of malignancies, which have been one of the leading causes of mortality. The early clinical diagnosis of cancer when it is confined to the organ(s) can greatly increase the expectation of complete patient recovery. As is well known, as tumors develop, the cells or the organ can release specific proteins into the circulation system. The levels of these proteins as tumor-related antigens in serum are associated with the stages of tumors. They can therefore be used as tumor markers for screening and clinical diagnosis of cancer. Reliable, sensitive tests of these tumor markers and a desirable evaluation system are very significant in early clinical diagnosis and evaluating the recovery of patients with certain tumor-associated diseases [1]. The need for reliable diagnostic tests for the rapid detection of tumor markers in human serum has attracted the efforts of the scientific community.

Methods and strategies based on biochemistry, immunology and molecular biology have been developed and used for

the determination of tumor markers in human serum. Immunoassay techniques have become the dominant test methods in clinical quantitative detection of tumor markers, due to the highly specific molecular recognition of antibody and epitopes of an antigen, and are mainly: radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay, chemiluminescent immunoassay, and electrochemiluminescent immunoassay. Although these detection techniques provide advantages (e.g., sensitivity, precision and selectivity), they often suffer because they present radiation hazards, take a long time, have high cost, and require qualified personnel and sophisticated instrumentation. Furthermore, existing diagnostic tests (e.g., microtiter-plate ELISAs) are not sensitive enough for screening cancer at an early stage and can only detect proteins at levels corresponding to advanced stages of disease. Optical detection often suffers from interference from other luminescence molecules. Also, single-analyte immunoassay is not fast enough for high-volume work. Further development of new immunoassay systems for sensitive, selective, low-cost, multi-analyte and high-throughput immunoassay has therefore attracted considerable interest.

Immunosensors, miniaturized analytical devices that combine high specificity of immunological reaction with sensitivity and convenience of detection techniques, have been developed rapidly as alternatives. Compared with traditional immunoassay methods, immunosensors are specific, simple and convenient, and can offer multi-target analyses and miniaturization. They

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can perform in situ, real-time, automation detection. Furthermore, their characteristics can satisfy the requirements of point-of-care testing (POCT) and bring molecular diagnostic assays to community health-care systems and underserved populations.

The significance and the development of immunosensors in cancer diagnosis have been summarized [2–6]. However, immunosensors continue to be associated with many other technologies, such as microarrays and nanotechnology. This review of immunosensors mainly focuses on recent development for tumor testing, applications as tumor markers and the outlook in clinical diagnosis.

2. Tumor markers

Table 1 lists the basic types of tumor markers, which are cellular products that are over-expressed by malignant cells. They include DNA, DNA modifications, RNA, proteins and protein modifications. Molecular tools, including both genomic and proteomic methods, are being developed to measure molecular alterations in the process of tumor growth. Although genomic methods (e.g., Southern blotting, bisulfite genomic DNA sequencing, PCR-amplification detection and DNA-microarray assays) could offer vast amounts of useful data in studying the phenomenon of DNA methylation, they suffer from being complex, laborious, time-consuming and not rigorous enough for clinical applications [7,8]. Furthermore, genetic markers cannot give information regarding post-translational modifications of proteins, so protein-based methods are being significantly developed. In this article, we focus on the development of protein-based immunosensors.

Tumor markers exist in blood at trace levels in the absence of a tumor. Upon the formation of a small tumor, the levels of some markers rise, so the limits of

detection (LODs) of the given methods are important for early screening of a small tumor. Table 2 gives the thresholds of basic tumor markers in human serum. However, most markers are not specific to a particular tumor. The specificity and the sensitivity are not normally ideal (e.g., CEA is associated with colorectal cancer, liver cancer and pancreatic cancer). Also, most cancers have more than one marker associated with their incidence, as shown in Table 3. Moreover, the levels of all tumor markers corresponding to the same tumor may increase in the serum of patient, so the use of panels of tumor markers can improve their diagnostic

Table 2. Normal levels for basic tumor markers

Tumor markers	Thresholds	Tumor markers	Thresholds
NSE	12.5 mg/l	CEA	3 µg/l
PSA	4 µg/l	CA125	35 kU/l
GST	3.2 U/l	CA153	25 kU/l
ALP	~0	CA27-29	36.4 kU/l
CT	100 ng/l	CA549	11 kU/l
SCCA	1.5 µg/l	CA19-9	37 kU/l
Ferritin	250 µg/l	CA50	14~20 kU/l
hCG	5.0 IU/l	CA242	20 kU/l
AFP	10 µg/l	CA72-4	6 kU/l

Table 3. Cancers and tumor-associated tumor markers

Cancer	Associated tumor markers
Liver cancer	AFP, CEA
Colorectal and pancreatic cancer	CEA, CA19-9, CA24-2
Gastric carcinoma	CA72-4, CEA, CA19-9
Esophagus carcinoma	SCC
Lung cancer	CEA, CA19-9, SCC, CYFRA21-1, NSE
Breast cancer	CA15-3, CA125, CEA
Epithelial ovarian tumors	CA125, AFP, hCG, CEA
Trophoblastic cancer	SCC, hCG
Prostate cancer	F-PSA, PSA, PAP

Table 1. Basic types of tumor markers

Types of tumor markers	Tumor markers
Enzyme	Neuron specific enolase (NSE); lactate acid dehydrogenase (LAD); aldolase; placental alkaline phosphatase (placental ALP); prostatic acid phosphatase; ribonuclease; 5'-nucleotidase; creatine kinase; γ -glutamyltranspeptidase (γ -GT); glutathione-S-transferase (GST); glycosyltransferase; aryl sulfatase B
Glycoproteins	Carcinoembryonic antigen (CEA); alpha-fetoprotein (AFP); human chorionic gonadotropin (hCG); prostate specific antigen (PSA); tissue polypeptide antigen (TPA)
Mucinous glycoproteins	CA125; CA15-3; DN-PAN-2; CA19-9; MCA; CA549; CA50; CA195; M26; M29; CA24-2; CA72-4
Hormones and related molecules	Ectopic hormones; calcitonin (CT); thyroglobulin; catecholamines; adrenocorticotrophic hormone; hormones of digestive tract; antidiuretic hormone; parathyroid hormone; prolactin; growth hormone
Molecules of the immune system	β_2 -microglobulin
Miscellaneous	α_2 -pregnancy-associated globulin; polyamines; ferritin; hydroxyproline; oncogene products; neurophysin; α_2 -acid glycoprotein; squamous cell carcinoma antigen (SCCA)
Acute-phase reactants	α_1 -antitrypsin; α_1 -antichymotrypsin; ceruloplasmin; c-reactive protein; haptoglobin; fibrinogen
Oncogenes	ras-oncogene (ras); myc-oncogene (myc); erbB-2/neu-oncogene (erbB-2); p53-oncogene (p53); bcl-oncogene (bcl)
Others	Microelement; fucose; hormone receptor; transferrin receptor; monoclonal antibodies to human tumor; immune complexes; lymphokines

value. The accurate multi-analyte test of combinations of tumor markers will affirm the result of the diagnosis of certain types of tumor, so multi-analyte immunoassays and immunosensing arrays for tumor markers are becoming an interesting and promising field of research.

3. Immunoassay

Immunoassay has been the main technique for detecting tumor markers in the past 40 years. There are different schemes of immunoassay in detecting tumor markers. The most popular formats are sandwich and competitive binding methods (Fig. 1). Since the measurable signal is difficult to obtain from an immunoreaction, a tracer is generally used to produce the analytical signal for estimating the occurrence of immunoreaction. A large variety of tracers, including radioisotopes, fluorophores and enzymes, have been applied in immunoassays.

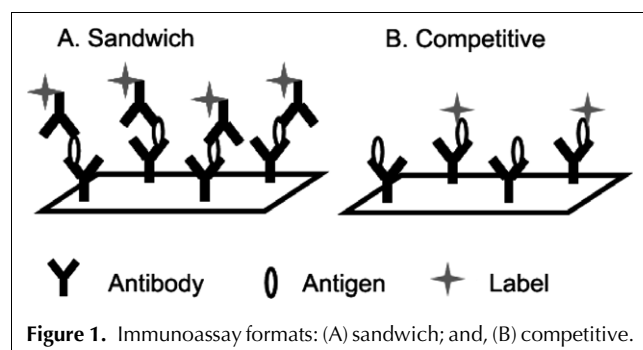
In sandwich assay, the immobilized antibody first captures the corresponding analyte (antigen), and the captured antigen is then detected by another labeled antibody (Fig. 1A). Sandwich assay has the advantages of high specificity and high sensitivity because of the use of two matched antibodies.

In competitive assay, antigen in the sample competes with labeled antigen to react with the immobilized antibody, and the antigen can be detected from the signal resulting from the labeled antigen (Fig. 1B).

Compared with the sandwich assay, the competitive assay is simpler and reduces incubation time. However, it suffers from a narrower linear range.

4. Immunosensors

Immunosensors are affinity ligand-based biosensing devices that couple immunochemical reactions to appropriate transducers. They can greatly simplify immunoassay, decrease the cost of clinic diagnosis and can miniaturize and automate detection. Furthermore, they can facilitate POCT and realize state-of-the-art molecular analysis without the need for a state-of-the-art laboratory.



Immunosensors can be classified as electrochemical, mass-sensitive and optical, according to their sensing principle.

4.1. Electrochemical

Electrochemical immunosensors combine antibody-antigen interaction with electrochemical measurements and they have been developed using different techniques (e.g., potentiometric, amperometric, impedance, capacitive, and conductometric).

Potentiometric immunosensors, based on the surface charge or potential change upon immunoreaction on the interface of the detection device, usually use a non-labeled technique. Liang et al. [9] developed a potentiometric immunosensor for the determination of breast cancer antigen (CA15-3) by utilizing glutaraldehyde to link CA15-3 antibody on a functional sol-gel film. This immunosensor showed a low LOD (5 U/ml) and satisfactory stability in a storage period of 30 days. However, the application of the potentiometric immunosensors is limited by non-specific binding between heterogeneous antigen and antibody and the influence of the high background signal.

Electrochemical-impedance immunosensors, based on impedance measurements of the electrical equivalent circuit of the oscillator, can characterize the electrical properties of immunoassay systems non-destructively without the need for reagents and a separation step. Zhu et al. [10] fabricated a biosensor for detecting CEA using Faradic impedance spectroscopy. CEA antibody was immobilized on gold-nanoparticle-modified glassy-carbon electrode by physical absorption. The tests revealed significant changes in the electron-transfer resistance of the $\text{Fe}(\text{CN})_6^{3-/4-}$ probe as the concentration of CEA changed.

Capacitive immunosensors are based on altering electrical conductivity at a constant voltage, caused by immunoreaction that specifically generates or consumes ions. Fernandez-Sanchez et al. [11] developed a disposable, non-competitive capacitive immunosensor for PSA by integrating a single-step, lateral-flow, immunoassay and impedance detection of the specific affinity event using an electrochemical transducer coated with a pH-sensitive polymer. This approach was particularly useful for designing a rapid, single-use immunosensor for sensitive detection of PSA.

They also prepared a quick, quantitative, disposable, impedance immunosensor for f-PSA and t-PSA [12]. Although this measurements of the immunosensor were inferred from impedance, it used capacitance measurement, ultimately. Because they can offer direct, label-free measurements, capacitive immunosensors are attractive for immunoassay and clinic diagnosis.

Amperometric immunosensors are among the most promising and most interesting immunosensors. They are usually designed to measure current generated by

electrochemical reaction. Since most analytes (proteins) cannot intrinsically act as redox partners in an electrochemical reaction, an electrochemically-active label is needed for the electrochemical reaction of the analyte at the sensing electrode. Enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), are the active labels most commonly used to catalyze the reaction of substrates to form electroactive products. Ju and co-workers [13–17] have made a great effort to develop amperometric immunosensors for tumor markers. Based on an electrochemical enzyme-linked immunoassay, using immobilized thionine as electron-transfer mediator between the electrode and the HRP-labeled anti-CEA antibody, they proposed an amperometric immunosensor for CEA with two linear ranges (0.6–17 ng/ml and 17–200 ng/ml) [13]. This sensor showed good accuracy and could be used for CEA detection in sera, with good agreement with the results obtained from the IRMA method. This method avoided addition of electron-transfer mediator to the solution, but the detection procedure needed an elution step of 10 min to elute the HRP-labeled antibody from the wells.

To simplify the assay system, a strategy based on the immobilization of carbohydrate antigen 19-9 (CA19-9) with titania sol-gel on a graphite electrode by vapor deposition was presented to prepare an amperometric immunosensor for CA19-9 [14]. The immobilized HRP formed in a competitive immunoreaction catalyzed the oxidation of catechol by H_2O_2 to produce amperometric signal for measurement of serum CA19-9. This is an example of “antigen-down” assay, as opposed to “antibody-down” described in the majority of the review. Using “antigen-down” in a competitive-immunoreaction format avoids using labeled-antigen.

Afterwards, a CEA immunosensor was fabricated by co-immobilizing thionine and HRP-labeled CEA antibody covalently on a glassy-carbon electrode with glutaraldehyde linkage [15]. The immunoassay was carried out by monitoring the decrease in catalytic efficiency of HRP, which was partly inhibited after the immunoreaction. This assay format not only got rid of adding electron-transfer mediator to the solution, but also avoided elution and washing steps.

Another way to simplify the procedure of amperometric immunoassay is to use direct electron transfer of labeled HRP, which can be carried out by measuring the direct electrochemical signal of bound HRP with regard to the Fe(III) to Fe(II) conversion without need for peroxide or mediator. Dai et al. [16] first introduced the new mechanism for amperometric immunoassay of tumor markers. The active center of bound HRP could exchange electrons with the electrode directly, so Dai et al. developed a reagentless, mediatorless immunosensor for detecting CA125, based on direct electrochemistry of labeled HRP. As shown in Fig. 2, the immunosensor was prepared by immobilizing CA125

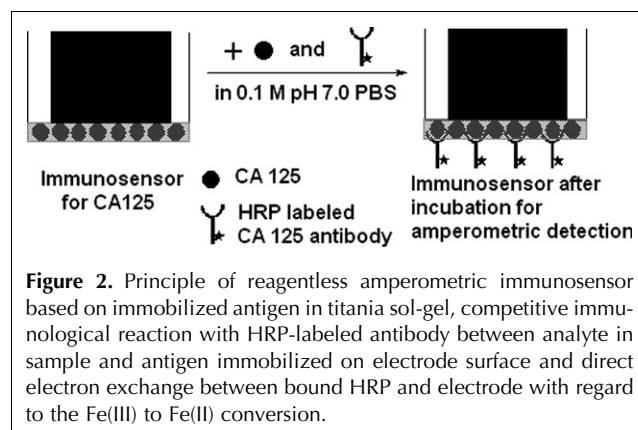


Figure 2. Principle of reagentless amperometric immunosensor based on immobilized antigen in titania sol-gel, competitive immunological reaction with HRP-labeled antibody between analyte in sample and antigen immobilized on electrode surface and direct electron exchange between bound HRP and electrode with regard to the Fe(III) to Fe(II) conversion.

with titania sol-gel on a glassy-carbon electrode by vapor deposition. After incubating the immunosensor in PBS containing HRP-labeled CA125 antibody and analyte, the HRP-labeled CA125 antibody was competitively conjugated with the immobilized CA125, leading to the formation of an HRP-modified surface. Based on the direct exchange of electrons between the immobilized HRP and electrode to produce detectable current signal, CA125 could be determined in the linear range 2–14 U/ml with an LOD of 1.29 U/ml.

This strategy avoided the trouble of adding a mediator to the sample solution and the washing step, and it provided a new platform for clinical assay. However, the detection range was limited due to the competitive assay format. Recently, this group [17] designed a reagentless CEA immunosensor, based on direct electrochemistry of labeled HRP, which was partly inhibited after the immunoreaction. The detection range of the CEA was 0.5–3.0 ng/ml and 3.0–120 ng/ml, and the LOD was 0.4 ng/ml, which was sufficient to measure clinically-relevant CEA levels (>3.0 ng/ml). Five serum samples from different individuals were used to evaluate the precision of this immunosensor, and the results showed acceptable agreement with the measurements from the analyzer used commonly in clinic laboratory.

Besides these reagentless immunosensors, based on the direct electron transfer of the labeled enzyme, Kerman and co-workers [18] presented a label-free electrochemical immunosensor for hCG based on the direct electrochemical response of the antibody and monitoring the change in the current signal of antibody upon the antigen binding. Their work avoided the use of labeled antibody and established an effective platform for developing simple, rapid, cost-effective amperometric immunosensors.

Screen-printing (thick-film) technology has attracted increasing interest in the past few years. Immunosensors based on screen-printed electrodes challenge conventional electrochemical immunosensors for disposability and portability.

Guan et al. [19] introduced an AFP immunosensor using Prussian Blue deposited on a screen-printed carbon electrode to catalyze electrochemical reduction of H_2O_2 produced from the enzymatic reaction of glucose oxidase. Based on a one-step sandwich ELISA, the detection range 5–500 ng/ml AFP was sufficient to measure clinically-relevant AFP levels ($>10 \mu\text{g/l}$). When real serum-sample testing was carried out using both this method and a typical ELISA, they showed similar results.

Ju et al. [20] proposed another strategy for preparing a disposable amperometric immunosensor for AFP based on enzyme-labeled antibody/chitosan membrane-modified screen-printed carbon electrode. The immunosensor was prepared by entrapping HRP-labeled AFP antibody in a chitosan membrane to modify the screen-printed carbon electrode.

Ju et al. [21] developed a CEA immunosensor based on CEA/colloid Au/chitosan membrane-modified screen-printed carbon electrode. As shown in Fig. 3, the immunosensor was inserted in a flow system with an injection of sample and horseradish peroxidase (HRP)-labeled CEA antibody. The CEA immobilized on the immunosensor trapped the labeled antibody to produce detectable current signal upon injection of substrates. Since the system was capable of continuously carrying out all steps, including incubation, washing, enzymatic reaction and determination, this method had the advantages of miniaturization, portability, and programmable operation without the need for a skilled operator, so it appears to have commercial potential.

4.2. Mass sensitive

The study of mass-based immunosensors mainly focuses on microbalances of piezoelectric crystals. The piezoelectric quartz-crystal device comprises a quartz-crystal wafer with different thicknesses sandwiched between two metal electrodes, which connect the device to an external oscillator circuit. The resonant

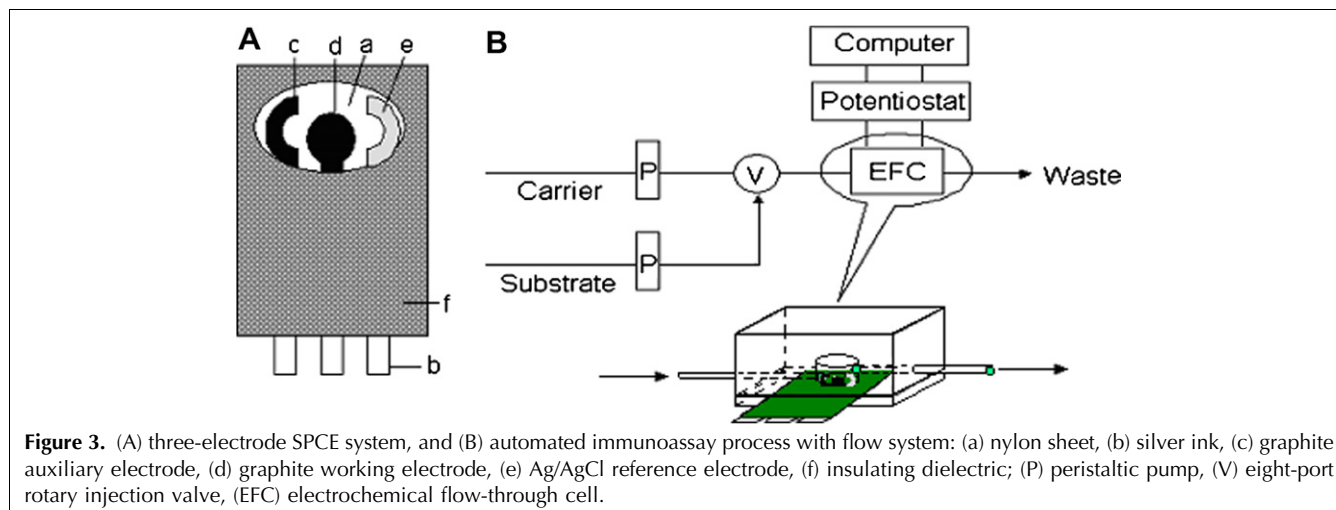
frequency of quartz crystal depends on the mass of the crystal surface as well as the mass of any layer confined to the electrode area of the crystal. The change of crystal frequency can reflect a tiny change in mass on the electrode surface.

Currently, piezoelectric immunosensors are used more increasingly to determine tumor markers in clinical diagnosis due to their advantages (label-free and real-time detection). Chou et al. [22] proposed a piezoelectric immunosensor for human ferritin by immobilizing anti-human ferritin antibody on a gold disc of a quartz-crystal microbalance. Human ferritin could be determined in the linear range 0.1–100 ng/ml. Zhang's group [23] presented a multi-channel, 2×5 model of a piezoelectric quartz micro-array immunosensor for quantitative detection of hCG in serum or urine samples. Compared with a one-channel immunosensor, this 2×5 model could detect hCG eight times faster.

In recent years, microcantilever sensors, another kind of mass-based sensor, have attracted much attention [24]. Microcantilever sensors can sensitively monitor the molecular adsorption, which bends the microcantilever and changes its resonant frequency. If two surfaces of the cantilever are chemically different, different molecular adsorption will produce different surface stress between top and bottom surfaces of the cantilever. When specific binding of biomolecules occurs on the surface of microcantilever, intermolecular nano-mechanical force induces the cantilever to bend and this can be observed as changes in cantilever deflection.

Microcantilever sensors do not require any label or reporter molecule to signal the presence of a molecule on a biosensor surface. In particular, nano-mechanical sensors have the advantage of high sensitivity with small area compared with other label-free biosensors (e.g., the quartz-crystal device).

Wee et al. [25] prepared a label-free PSA immunosensor using self-sensing piezoresistive microcantilevers.



Electrical detection of antigen-antibody specific binding was accomplished through a direct nano-mechanical response of micro-fabricated self-sensing micro-cantilevers. This cantilever immunosensor was used for the detection of PSA and C-reactive proteins and was shown to be effective for clinical application.

Wu and co-workers [26] developed a label-free immunosensor for PSA with microcantilevers using a polyclonal antiprostata antibody as a covalent linker. In this system, the PSA could be detected in the concentration range 0.2–60 µg/ml.

4.3. Optical

Optical immunosensors, including fluorescence [27,28] chemiluminescent [29–33] and surface-plasmon resonance (SPR) [34–40] types, have mainly been considered for immunoassay due to their advantages in applying visible radiation, non-destructive operation, and rapid signal generation and reading.

In recent years, fluorescent detection methods have achieved major improvements in clinical applications. Nakamura et al. [27] fabricated an hCG immunosensor coupled to a flow-immunoassay system and a cation-exchange resin-packed capillary column. The hCG and fluorescein isothiocyanate (FITC)-conjugated IgG antibody complex was separated from free FITC-conjugated IgG on the basis of difference in isoelectric point. The fluorescence intensity correlated linearly with the concentration of hCG in the range 25–1500 mIU/ml.

Time-resolved fluorimmunoassay (TRFIA) is a highly sensitive fluorescent detection method. For minimal reagent consumption, rapid separations, and automation, Yan et al. [28] developed a time-resolved fluorometric immunosensor for CEA determination coupled to a flow-injection system (Fig. 4). The prepared immunoaffinity column was inserted in a flow system for immunoreactions. After the enhancement solution was injected to cleave the Eu labels from the immunocomplex, the cleaved solution was collected and detected by time-resolved fluorescence.

Chemiluminescent immunosensors combining antibody-antigen interaction with sensitive chemiluminescent measurements are popular optical immunosensors. There are three major chemiluminescent analytical techniques, using direct chemiluminescent tags, chemiluminescent substrate labels and enzyme labels respectively.

The direct chemiluminescent tags label chemiluminescent reagents (e.g., acridinium ester and acridinium sulfonamide ester) to antibody or antigen. After immunoreaction, the labeled reagents can react with peroxide without addition of enzyme to emit light. When chemiluminescent substrates (e.g., luminol, isoluminol and dioxetane phosphate ester) or enzymes (e.g., HRP and alkaline phosphatase) are used to label to antibody or antigen, the enzymatic reactions to convert substrates to

luminescent products produce detectable signal after immunoreaction. These signals are related to the concentration of antigen or antibody.

Most chemiluminescent immunosensors use enzymes to amplify the detection signal. Sasamoto's group [29] fabricated a chemiluminescent immunosensor for hCG using phenacyl phosphate as a chemiluminescent label. However, this immunoassay was performed in microtubes, and resulted in time-consuming, complex handling.

The trend in chemiluminescent immunoassay is towards automation. Ju's group [30–33] made the effort to automate the detection procedures of chemiluminescent immunoassay using a flow-injection system. They introduced a non-competitive enzyme immunoassay for CEA using flow-injection chemiluminescence [30]. A CEA-immobilized immunoaffinity column was used in the flow system to trap the unbound HRP-labeled antibody after off-line incubation of analyte and HRP-labeled anti-CEA. The HRP-labeled antibody bound in the immunocomplex could be detected by injecting substrates to enhance chemiluminescence, which was proportional to the concentration of the analyte in human serum.

They prepared an AFP immunosensor by detecting the enzyme activity of the unbound HRP-labeled antibody trapped in the immunoaffinity column [31].

With a similar immunoassay scheme, they fabricated an immunosensor for CA19-9 by immobilizing antigen on a cross-linked chitosan membrane [32].

Ju et al. [33] developed a chemiluminescent immunosensor for AFP based on epoxysilane-modified glass microbeads, which provided a rigid material with good transparency. With a non-competitive immunoassay format, the proposed immunosensor system enabled a low-cost, flexible, rapid, quantitative assay for AFP.

SPR uses an evanescent wave phenomenon to detect change in the refractive index of the surface medium. SPR-based immunosensors offer label-free, real-time assays for a wide range of molecules (e.g., proteins, complex conjugates, toxins, allergens, drugs and pesticides) with low LODs in the range 10^{-9} – 10^{-13} M [34].

Chou's group [35] developed an SPR immunosensor for human ferritin, a non-specific tumor marker, based on the immobilization of anti-human ferritin monoclonal antibody on an SPR-sensing gold surface.

Campagnolo et al. [36] reconfigured a refractometer to detect tumor molecular interactions through changes in SPR signal.

SPR immunosensors have also been successfully commercialized. Huang et al. [37] introduced a PSA immunoassay using a commercially-available SPR biosensor, based on mixed, self-assembled monolayers.

In recent years, many efforts have been made to overcome the disadvantage of inherent low sensitivity for detecting small molecules. Besselink et al. [38] amplified the assay sensitivity for PSA in SPR with col-

loidal gold nanoparticles (10-nm diameter) and latex microspheres (120-nm diameter) on planar-type and gel-type sensor surfaces. The corresponding LOD for PSA was approximately 0.15 ng/ml, which was sufficient for measuring clinically-relevant PSA levels (>4 ng/ml).

Yu and Knoll [39] proposed an immunosensor for hCG based on the diffraction of surface plasmon. The inherent self-referencing mechanism of surface diffraction was found to be very effective in compensating for fluctuations of the bulk. The majority of existing SPR-sensing devices offer only a small number of sensing channels (<10), so their throughput is rather limited. However, Piliarik's group [40] proposed a high-throughput SPR sensor based on combination of SPR imaging with polarization contrast and a spatially patterned multilayer SPR structure.

5. Multi-analyte immunosensing

In clinic diagnosis, measurement of a single tumor marker often has limited diagnostic value because most markers are not specific and sometimes they can have elevated levels in patients without cancer. However, most cancers show elevation of the level at least two markers associated with their incidence. Simultaneous multi-analyte immunoassays (SMIAs) of multi-tumor markers associated with a particular type of cancer may confirm the results of diagnostic screening. Furthermore, SMIAs are a promising analytical method for protein analysis with the advantages of short analysis time, simple analytical procedure, small sample volume, test efficiency and lower cost than parallel single-analyte assays. The short analysis time and simple procedure make these technologies useful for high-throughput detection.

There are two main modes for SMIA of multi-tumor markers [41], one of which comprises multiple-label assays.

Matsumoto and co-workers [42] reported a simultaneous measurement method for alpha-fetoprotein (AFP) and CEA in human sera by time-resolved fluoroimmunoassay. In this assay, they combined Eu-labeled anti-AFP antibody and biotinylated anti-CEA antibody complexed to Sm-labeled streptavidin for CEA, and the LODs were 0.07 ng/ml for AFP and 0.3 ng/ml for CEA. The sensitivity was sufficient for multi-analysis of clinically-relevant AFP and CEA levels. To evaluate the accuracy, AFP and CEA concentrations in 27 human sera were determined with both the simultaneous measurement method and the typical single-assay method in clinic diagnosis, and the results showed that the proposed method had high precision.

Zhang and co-workers [43] developed a dual-label immunoassay method for the simultaneous determination of AFP and hCG. by using Eu³⁺-labeled anti-AFP

and Sm³⁺-labeled anti-hCG under a sandwich immunoassay format.

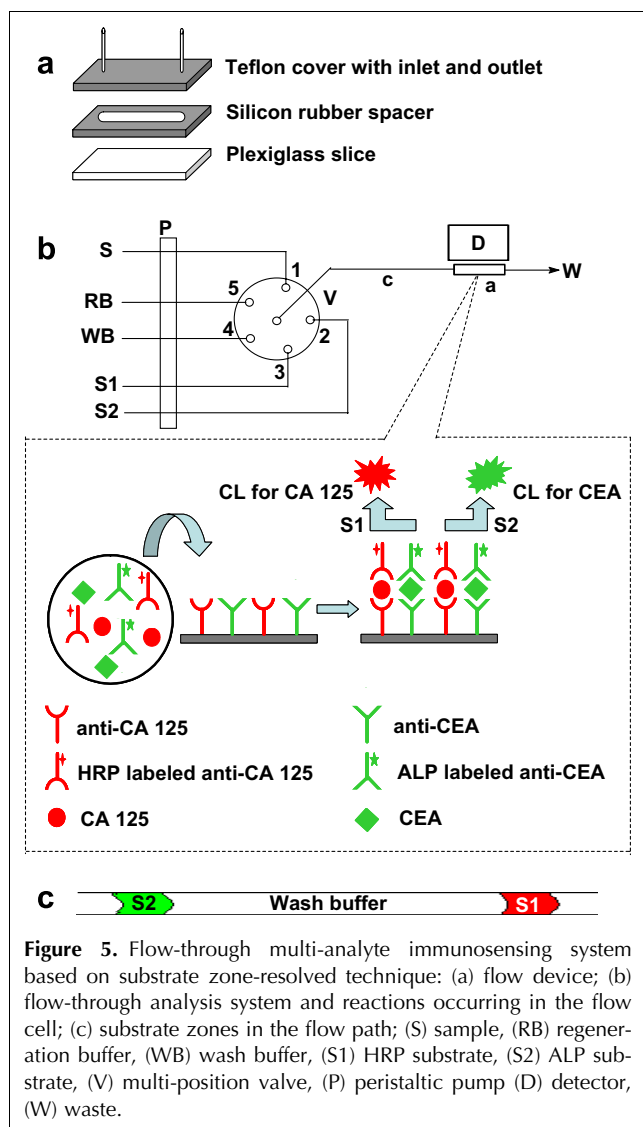
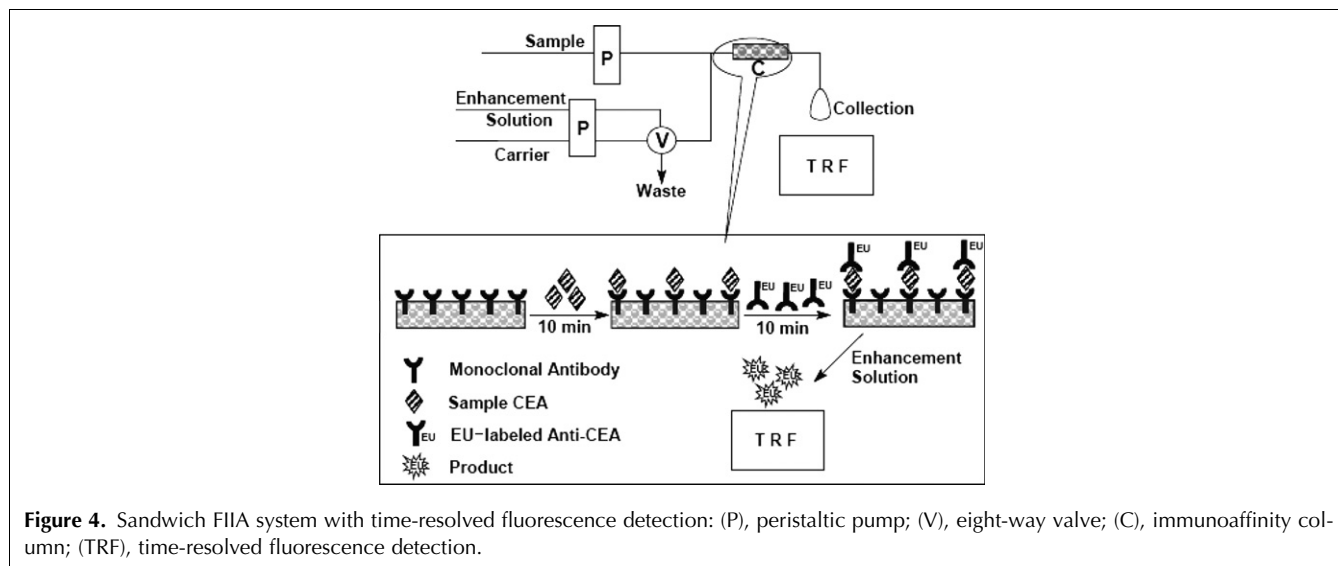
Katsutoshi et al. [44] proposed a simultaneous TR-FIA for the determination of AFP, hCG and estriol, which are important substances for Down's syndrome, using europium and samarium ion chelate as labels. In this assay, a 96-well microtiter plate was used for the AFP and hCG assay and a transferable solid-phase plate was used for the estriol assay. They used a combination of multi-label mode and spatially-resolved methods that extended previous concepts of one label for one analyte in a multi-label assay. Unfortunately, multi-label assays have a limited number of labels and need a compromise for assay conditions, and that leads to lower sensitivity than a single-assay technique.

Another assay format, based on spatially-separated test zones, has gained more consideration recently. Using plastic capillaries and fluorescent detection, a spatially-separated multi-analyte immunosensor for the determination of follitropin, hCG and prolactin has been fabricated by coating different antibodies on the discrete areas of the internal capillary surface [45].

Song et al. [46] developed a simultaneous multiplexing immunoassay for six tumor markers by using antibody fragments in a microarray system. The sandwich format produced a wide calibration range for CEA, AFP, β -HCG, CA 125, CA 19-9 and CA 15-3.

Cross-reactivity between analytes and non-cognate antibodies and cross-talk (referring to signal interference) are two main factors for spatially separated immunosensor array. To avoid signal interference, Ju's group [47] proposed a flow-through, multi-analyte, immunosensing system based on a substrate zone-resolved technique for two tumor markers. As shown in Fig. 5, using CA125 and CEA as model analytes, the CA125 and CEA antibodies were immobilized on an UltraBind aldehyde-activated membrane as an immunoreactor to capture CA125, CEA and their corresponding tracers, HRP-labeled anti-CA125 and ALP-labeled anti-CEA, in a flow cell. The substrates for HRP and ALP were then delivered into the detection cell sequentially to perform substrate zone-resolved immuno-assay using a sandwich format. The substrate zone-resolved multi-analyte analytical technique could obtain distinguishable CL signals without consideration of wavelength, and provide each CL reaction catalyzed by the label in its optimal assay condition without loss of assay performance. This method could control all steps by automation. The short test time of 35 min made possible high sample throughput.

In electrochemical multi-analyte immunosensors, Kojima et al. [48] resolved the cross-talk by considering sufficient separation between sensing electrodes and the immobilizing matrix. They micro-fabricated the protein chip to detect AFP and β_2 -microglobulin by immobilizing capture antibodies in a double siloxane



layer, which could delay diffusion of enzyme-generated product.

Wilson [49] used microfabrication technology to prepare an electrochemical immunodevice comprising two iridium-oxide (IrOx) sensors for the simultaneous detection of CEA and AFP. Because the IrOx matrix could also delay diffusion of electroactive product generated by enzyme action, it enabled the highly efficient capture of electroactive product on the electrode, reducing signal interference, and showed the potential for fabricating smaller devices with less distance between electrodes. On this basis, they further developed an array-based immunodevice including eight IrOx electrodes for simultaneous detection of four different proteins [50] and seven tumor markers [51].

Besides electrochemical systems, real microelectrode arrays have also been developed. Using CombiMatrix microarrays, a high-density electrode array, providing over 1000 electrodes/cm², has been used to develop spatially-multiplexed assay formats for five analytes [52]. This array could measure biological entities over a wide range of sizes, from small molecules to cells.

6. Nanotechnology for immunosensors

The emergence of nanotechnology has opened up new horizons for molecular diagnostics [53]. Nanoparticles, such as gold colloids and quantum dots (QDs), have been widely used for fabricating immunosensors based on their advantages of high surface-to-volume ratio, more freedom in orientation for protein immobilization, facility in electron transfer, size-tunability, and high quantum yields.

Ju et al. [54] fabricated an amperometric immunosensor by immobilizing colloidal gold nanoparticle-modified CA125 in a cellulose acetate membrane

based on the good biocompatibility and the high stability of colloidal gold. It could determine CA125 in the linear range 0–30 U/ml with an LOD of 1.73 U/ml.

Another reagentless hCG immunosensor, based on encapsulating immunoconjugate in a gold nanoparticle/titania sol-gel composite membrane was also developed [55]. This composite architecture showed an efficient scaffold of nano-scale dimension with an orderly structural organization of a large variety of nanoparticles. The existence of gold nanoparticles not only provided a very hydrophilic interface for retaining the bioactivity and improving the stability of the immobilized enzyme labels and immunocomponents, but also promoted electrical communication between redox sites of enzyme labels and sensing surface for direct electrochemical immunoassay of protein or antigen analytes.

QDs based on narrow photoemission spectra, high resistance to photobleaching, and broad excitation spectra are widely used as tags in immunoassay. A CEA immunosensor has been fabricated using biofunctionalized QD probes [56]. This immunosensor array was designed to detect a wide range of analytes using the inherent characteristics of QDs and the flexibility of engineering elastin-like polypeptide fusion.

Beside these applications of nanoparticles, miniaturized multiplexed immunoassay nanodevices based on nanowires have attracted much attention. Zheng et al. [57] described an innovative approach for real-time, label-free, multiplexed electrical detection of four cancer markers using a silicon-nanowire array. They developed integrated nanowire arrays, in which distinct nanowires and surface receptors could be incorporated as individual device elements. Protein markers were routinely detected at femtomolar concentrations with high selectivity, and simultaneous control by nanowires discriminated against false positives.

7. Future outlook

The levels of tumor markers in human serum can give information about the stage of development of certain tumors in patients. Immunosensors, which involve biology, physical science, chemistry and iatrology, have potential for clinical testing because of their low cost, easy operation, rapid determination, high selectivity and good sensitivity. However, few of these technologies have transferred into the clinical arena, since they are in the development phase.

Searching for functional labels for proteins (e.g., amino-active fluorescent chelates that have high fluorescence quantum yields and long fluorescence lifetimes) has always been the goal in immunoassay. In addition, label-free immunoassay formats that employ SPR and quartz-crystal devices should be further developed. These

technologies allow direct measurement of biomolecular interactions, avoid labeling and provide real-time detection. Of course, all-important trends in immunosensors are their high throughput, miniaturization, point-of-care application and commercialization. Decreasing the volume of the immunoassay reaction chamber, improving detection techniques, reducing non-specific adsorption and realizing “lab on a chip” have become important goals.

For clinical diagnosis and diagnostic screening of cancer, SMIA are widely considered, and the availability of a wide range of nanotechnologies will also lead to development of miniaturized, multiplexed nanoimmunosensors with the potential to be very important as on-site diagnostic tools.

Analytical techniques with differences in time, space, substrates, reactants, labels and detection methods will be designed and developed for multi-analyte immunoassays. Screen-printed and advanced microtechnological approaches will increasingly attract interest in manufacturing immunosensors.

Integration into flow-injection systems, capillary electrophoresis and microfluidic platforms is just the latest, logical step in the direction of automation. All these trends are important and should occur in parallel in the future development of immunosensors and immunoassay methods for biomedical and clinical applications.

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