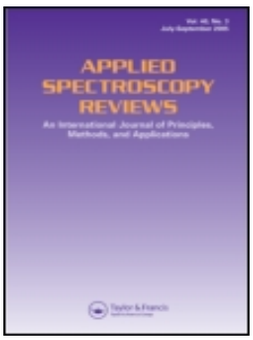

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Silver Enhancement of Gold Nanoparticles for Biosensing: From Qualitative to Quantitative

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Abstract: *The establishment and rapid progress in sensitive biosensing using immunogold silver enhancement has occurred in the past 30 years. Its high sensitivity and simplicity have made immunogold silver enhancement a revolutionary technique for signal amplification in biosensing. This review focuses on the major applications of immunogold silver enhancement, with special emphasis on quantitative biosensing. In this review, the combinations of immunogold silver enhancement with a series of quantitative techniques, such as colorimetry, electrical and electrochemical methods, gravimetry, chemiluminescence, Raman spectroscopy, and inductively coupled plasma–mass spectrometry (ICP-MS), are discussed in detail. Immunogold silver enhancement has become one of the most useful methods in highly sensitive quantitative bioanalysis. The recent development of ICP-MS detection shows great potential in combination with immunogold silver enhancement.*

Keywords: Silver enhancement, gold nanoparticles, biosensing, ICP-MS, amplification

Introduction

A highly sensitive technique for biomolecules sensing is important in the early diagnosis and elucidation of molecular mechanisms for many diseases, because even a few biomolecules is sufficient to affect the biological functions of cells and trigger pathophysiological processes (1). Unfortunately, using conventional methods, it is still hard to detect some important disease biomarkers, due to their extremely low abundance in body fluids or tissues (2). Hence, it is urgent to develop highly sensitive methods for the detection of bioanalytes with low abundance. Generally, high sensitivity can be obtained using signal amplification procedures. Many signal amplification schemes for sensitive bioanalytical detection have been reported, such as rolling circle amplification (3), avidin–biotin ampli-

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fication (4), liposome amplification (5), in-line atom transfer radical polymerization (6), and so forth. However, these methods usually require a series of complex reagents and tedious procedures. Due to its easy handling, inexpensive reagents, robustness, and high sensitivity, the immunogold silver enhancement technique has been widely used and even successfully commercialized since it was first developed in 1983 by Holgate et al. (7) and Danscher and Nørgaard (8). It is used as a sensitive and specific immunohistochemical visualization technique for qualitative biosensing. The use of immunogold silver enhancement is not restricted to qualitative immunohistochemical methods but can be combined with various quantitative techniques, such as colorimetry, electrical and electrochemical methods, gravimetry, chemiluminescence, Raman spectroscopy, and inductively coupled plasma–mass spectrometry (ICP-MS). There are some excellent reviews about the qualitative biosensing using this technique (9–12); thus, only a brief introduction is given in this review. The present review aims to present major developments in this state-of-the-art immunogold silver enhancement technique with emphasis on the applications in quantitative biosensing aspect. The combination of immunogold silver enhancement with a series of quantitative methods is introduced with specific examples. Moreover, the advantages and the limitations of this technique are discussed in detail.

The Basics of Silver Enhancement

Generally, the basic principles and processes of immunogold silver enhancement are as follows: First, silver ions adhere to the surface of the catalytic gold nanocrystal and become part of the crystal; then they are reduced to silver atoms by electrons released from the reducing molecules in solution around the gold nanocrystal (12). Because the silver atoms deposit on the surface of the original nanocrystal and possess the same catalytic capability, the silver enhancement process continues as long as there is an adequate supply of silver ions and reducing molecules in the vicinity of the silver grains, thus expanding the size of the nanocrystals. Using the principles of immunogold silver enhancement, a typical signal-amplified immunoassay is illustrated in Figure 1.

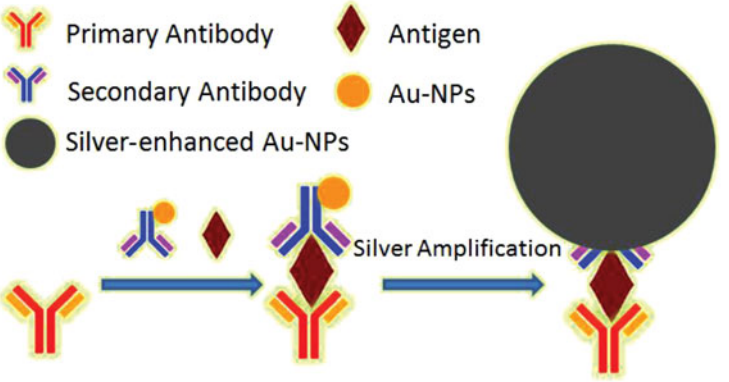

Figure 1. Typical immunogold silver enhancement–based immunoassay (66). Reproduced from (66) with permission from the American Chemical Society. (Color figure available online.)

A number of silver salts—that is, silver lactate (8, 13), silver nitrate (14), and silver acetate (15)—have been used as the sources of immunogold silver enhancement. Among them, silver nitrate and silver lactate are light sensitive and consequently the signal amplification procedure needs to be carried out in darkroom conditions or in a dark cupboard. In contrast, silver acetate is light-insensitive during immunogold silver enhancement. Thus, silver acetate is considered to be the best silver source for immunogold silver enhancement. The ingredient of silver acetate amplification was adopted from Hacker et al. (15) as follows: Citrate buffer (pH 3.5) was prepared by dissolving 23.5 g of trisodium citrate dihydrate and 25.5 g of citric acid monohydrate in 850 mL of deionized water. This buffer can be kept at 4°C for at least 2 to 3 weeks. Before use, it is adjusted to pH 3.8 with citric acid solution. Fresh silver amplification solutions (A and B) were prepared. For solution A, 80 mg of silver acetate was dissolved in 40 mL of deionized water (silver acetate crystals can be dissolved by continuous stirring within about 15 min); for solution B, 200 mg of hydroquinone was dissolved in 40 mL of citrate buffer. Solution A and solution B were mixed with an equal volume immediately before use.

Silver Enhancement of Gold Nanoparticles for Qualitative Biosensing

Gold nanoparticles are ideal markers in qualitative imaging biosensing systems for several reasons: First, they can be readily prepared in a wide range of sizes, from about 2 nm to greater than 100 nm in diameter; second, the specific activities of biomolecules can be retained when coupling to gold nanoparticles; third, the gold nanoparticles can be easily visualized within biological systems (organism, tissues, cells, etc.) under electron microscopy with high contrast. Since gold nanoparticles labeling techniques were first introduced in 1971 by Faulk and Taylor (16) to identify *Salmonella* antigens, they have been widely applied to both transmission electron microscopy and scanning electron microscopy. Gold nanoparticles labeling is used for its high electron density, which increases electron scatter to give high-contrast dark spots. They are most often attached to secondary antibodies, which are attached to primary antibodies designed to bind a specific protein or other cell component. It is especially useful in highlighting proteins found in low densities, such as some cell surface antigens. The immunogold labeling technique can be adapted to distinguish multiple objects using different sizes of gold particles. Usually, a thin section of the sample is prepared using a microtome. The prepared sample section is then incubated with a specific primary antibody designed to bind the molecule of interest. Subsequently, a gold nanoparticles–labeled secondary antibody is added, and it binds to the primary antibody. Gold can also be attached to protein A or protein G directly instead of a secondary antibody, because these proteins bind the Fc regions of IgG in a nonspecific way. The electron-dense gold nanoparticles can then be seen under an electron microscope as a black dot, indirectly labeling the molecule of interest.

However, the gold nanoparticle labels are often too small to be discernible under light microscope. The immunogold silver enhancement technique has proved to be an effective solution to this problem, which provide a sensitive amplification system by depositing metallic silver at the site of the gold, thus increasing its size and producing a label visible as a black product under bright field light microscopy. Although this process is a purely chemical reaction, the silver deposition is autocatalytic and the amplification and signal-to-noise ratio can equal or exceed that for catalytic deposition of chromogens by enzyme-labeled reagents (9). The immunogold silver enhancement technique is an important addition to the available range of immunohistochemical visualization methods. There are some excellent reviews relevant to silver-enhanced immunohistochemical visualization (9, 12, 17–25);

thus, only a brief introduction is provided here. Among these reviews, two tutorial reviews are recommended before performing silver enhancement experiments (9, 12).

Gold clusters, such as 1.4-nm nanogold, are gold compounds that can be covalently linked to Fab′ fragments of the antibodies, making small and stable probes. Improved properties and better experimental performance have been obtained, including higher labeling density, better sensitivity, and greater penetration into tissues (19). Silver enhancement makes these small gold clusters easily visible by electron microscope, light microscope, and even directly by eye. Chemical cross-linking of gold clusters to many biologically active molecules is possible using novel probes that are not feasible with colloidal gold.

It is worth mentioning that in addition to microscopy, some novel imaging technologies, such as laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS) can be combined with immunohistochemistry using immunogold silver enhancement. As an example, LA-ICP-MS was applied to the imaging of breast cancer–associated proteins (26). The method showed higher sensitivity compared to optical microscopy and acceptable resolution. As shown in Figure 2, an imaging run was conducted using a laser beam with diameter of 5 μm and a line raster rate of 5 μm s^{-1}. This resulted in an improved capability to map fine-scale features, although on close inspection it is clear that image quality and definition for LA remain inferior to that for microscopy. Respective images and line rasters for Au/Ag were in perfect correlation, and sensitivity was enhanced about 130 fold after silver amplification.

Silver Enhancement of Gold Nanoparticles for Quantitative Biosensing

Traditional immunohistochemical methods, although ubiquitous in both clinical and research settings, are only qualitative or semiquantitative approaches to biosensing (27). Quantitative biosensing is an important research field because many specific functions in a biological cell are controlled by changes in biomolecular expression levels under different physiological conditions. Consequently, analytical methods that allow the quantification of individual biomolecules synthesized by a cell at a given moment and under specific conditions are necessary. The thick silver shell on the gold nanoparticles endows particular features, such as colorimetry, electrical and electrochemical methods, and gravimetry for quantitative biosensing. Typical quantitative biosensing strategies using gold–silver enhancement are summarized in Table 1.

Colorimetric Method

Immunosorbent Assay. Enzyme-linked immunosorbent assay (ELISA) is a common analytical biochemistry assay that uses antibodies and color change to identify a substance (antigen). In ELISA, the enzyme reaction provides a colorimetric signal that indicates whether a specific antigen or antibody is present in the sample. Due to its high sensitivity, high specificity, and cost-effectiveness, ELISA is widely used as a routine method for the detection and quantification of hundreds of types of molecules native to both living organisms (e.g., hormones) and foreign molecules (e.g., pharmaceuticals). However, the enzymes are expensive and have a limited shelf life. A silver-enhanced, gold-labeled, immunosorbent assay for the detection of HIV antibodies in human serum sample was developed by Rocks et al. (28). From a methodological point of view, a silver-enhanced, gold-labeled, immunosorbent assay is similar to ELISA; the same technique and apparatus are used. The silver deposit can be read visually or colorimetrically. Further development has allowed the use of whole blood (29) and a screening assay for rubella antibodies (30). The method has

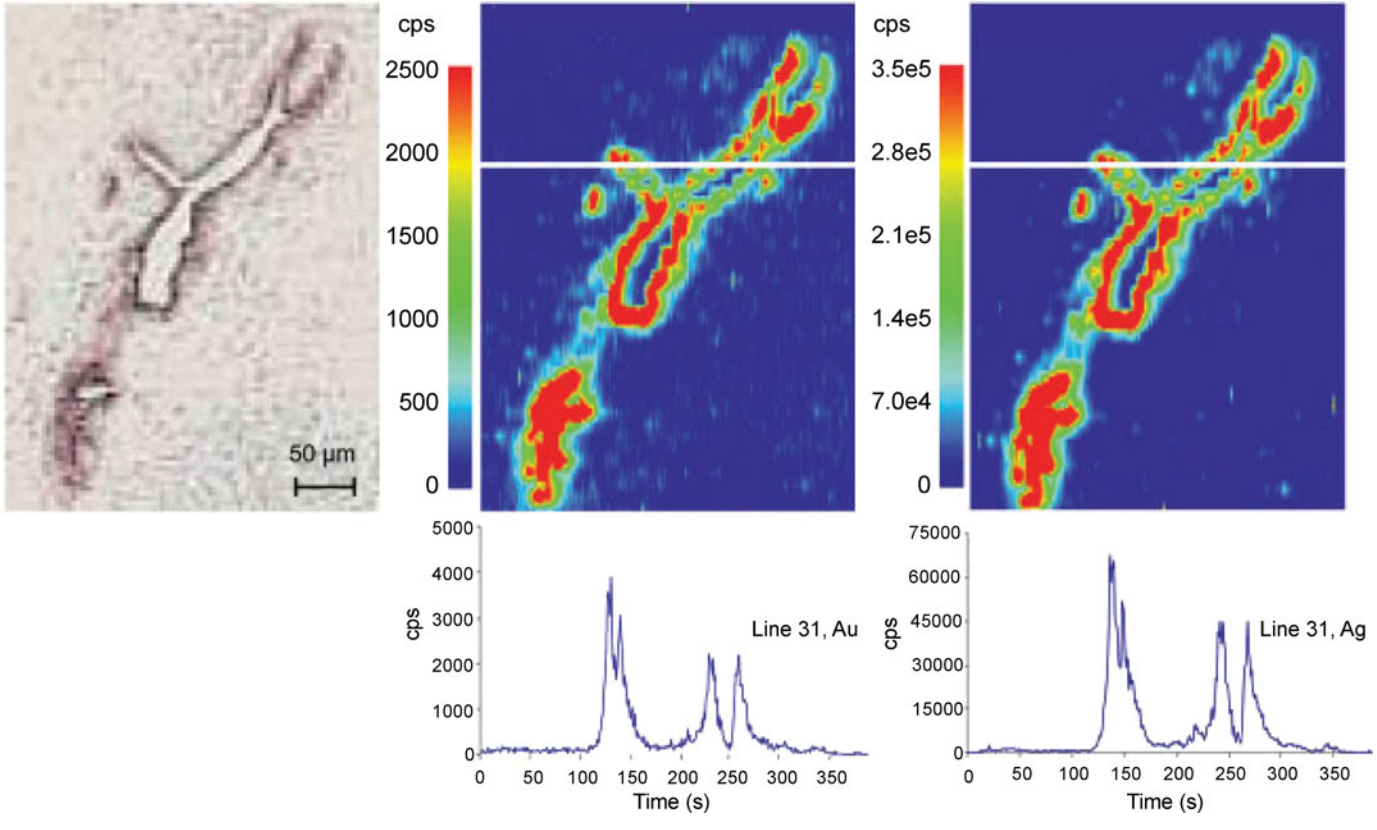

Figure 2. Photomicrograph of tissue section, corresponding Au distribution maps without Ag enhancement, and Ag distribution maps after Ag enhancement by LA-ICP-MS (47 line rasters) (26). Inset: single line raster for Au and Ag. Reproduced from (26) with permission from John Wiley & Sons. (Color figure available online.)

Table 1
Typical quantitative biosensing strategies using gold–silver enhancement

Detection method	Analyte	Diameter of Au nanoparticles (nm)	Sensitivity enhancement (-fold)	LOD	Reference
Colorimetric (microplate)	Human growth hormone	—	—	0.4 mIU L^{-1}	(67)
Electrochemical (voltammetry)	Streptavidin	10		2.0×10^{-15} M	(68)
Electrochemical (voltammetry)	DNA	20	83	0.2 ng mL^{-1}	(69)
Electrochemical (voltammetry)	DNA	16	100	50 pM	(45)
Electrochemical (voltammetry)	IgG	20	—	1 ng mL^{-1}	(46)
Electrochemical (voltammetry)	*Schistosoma japonicum* antibody	20	—	3 ng mL^{-1}	(14)
Electrochemical (voltammetry)	Specific DNA-binding protein	13	—	0.1 pM	(48)
Electrochemical (voltammetry)	DNA	Au/Fe_3O_4 nanocomposites	800	100 aM	(70)
Electrochemical (voltammetry)	Concanavalin A	10	—	0.07 μg mL^{-1}	(47)
Electrochemical (voltammetry)	DNA	13	—	72 pM	(49)
Electrochemical (voltammetry)	Carcinoma embryonic antigen	323 (Au nanoparticle–coated polybeads)	—	0.12 pg mL^{-1}	(50)
Electrical (resistance)	Bioconjugated Au NPs	30	10	—	(71)
Electrical (resistance)	DNA	13	—	500 fmol	(42)
Electrical (resistance)	Protein A antibody	—	—	10 ng mL^{-1}	(44)
Electrical (resistance)	Alpha-fetoprotein	10	—	1 ng mL^{-1}	(43)
Electrical (resistance)	Ricin toxin	10	—	10^{-11} M	(72)
Electrical microchip (resistance)	Protein A	20	—	1 ng mL^{-1}	(73)
Electrical microchip (resistance)	IgG	40	—	<240 pg mL^{-1}	(74)
Lateral flow immunoassay	Neurotoxin type B	25	1,000	50 pg mL^{-1}	(75)
Lateral flow immunoassay	Abrin-a	40	100	0.1 ng mL^{-1}	(40)
Lateral flow immunoassay	H5 influenza virus hemagglutinin	—	500	0.5 ng mL^{-1}	(41)

Scanometric method	Hg^{2+}	20	10	10 nM	(32)
Scanometric method	Concanavalin A	14	—	39 nM	(33)
Visual detection	Thrombin	13	—	14 fmol	(37)
Visual detection	Adenosine	16	—	0.1 pmol	(38)
Visual detection	*Ureaplasma parvum* and *Chlamydia trachomatis* antibodies	10–15	—	2 ng mL^{-1}	(39)
Colorimetric method	DNA	13	100	50 fM	(31)
Colorimetric method	Biotinylated DNA	10	—	0.1 fmol	(76)
Colorimetric method	IgG	—	—	2.75 ng mL^{-1}	(34)
Colorimetric method	IgG	10	—	89 pM	(77)
Colorimetric method	Polynucleotide	15	—	600–6,000 Molecules	(78)
Colorimetric method (densitometry)	IgG	5	—	0.1 μg cm^{-3} (total)	(79)
Colorimetric detection (dot-blot)	Biotin–peptide	20	1,000	100 zmol	(35)
Colorimetric detection	MicroRNA	15	—	10 fM	(80)
Colorimetric detection	IgG	10	—	0.05 ng mL^{-1}	(36)
Colorimetric detection	DNA	13	—	0.9 nM	(81)
Colorimetric detection	Lysozyme and adenosine 5-triphosphate	Polydimethylsiloxane Au nanoparticle composite film	—	1×10^{-4} μg mL^{-1}	(82)
Ultrasonic	Breast epithelial mucin antigen	10	—	0.01 μg mL^{-1}	(52)
Quartz crystal microbalance	IgG	10	100	—	(53)
Surface-enhanced Raman scattering spectroscopy	DNA, RNA	13	—	500 fmol	(55)
Resonance light scattering	Kemptide	13	—	<1 fg	(56)
Chemiluminescence	Human IgG	16	100	0.005 ng mL^{-1}	(54)
ICP-MS	Carcinoma embryonic antigen	12	60	0.03 ng mL^{-1}	(66)

also been applied to the competitive immunoassay of low-density lipoprotein and sandwich immunoassay of human growth hormone. The results were comparable with ELISA. A limit of detection (LOD) of 0.4 mIU/L was obtained for human growth hormone, compared to 0.3 mIU/L for ELISA. The use of immunogold silver enhancement allows an inexpensive immunoassay and does not require enzymatic or radioactive labels. The silver deposit can be read either visually or quantitatively on a conventional microplate reader. Moreover, the dried plate may be stored for future reference.

Scanometric Method. A silver-enhanced scanometric method for analyzing combinatorial DNA arrays using oligonucleotide-modified gold nanoparticle probes and a conventional flatbed scanner was developed by Taton et al. (31). Labeling oligonucleotide targets with gold nanoparticles rather than fluorophore probes substantially altered the melting profiles of the targets from an array substrate. This difference permits the discrimination of an oligonucleotide sequence from targets with single nucleotide mismatches with a selectivity over three times that observed for fluorophore-labeled targets. In addition, when coupled with silver enhancement, the sensitivity of this scanometric array detection system exceeded that of the analogous fluorophore system by two orders of magnitude. The silver-enhanced scanometric method was further fabricated for the detection of mercuric ion (Hg^{2+}) (32). The method takes advantage of the cooperative binding and catalytic properties of DNA-functionalized gold nanoparticles and the selective binding of a thymine–thymine mismatch for Hg^{2+}. The LOD of the assay in buffer and environmentally relevant samples (lake water) was 10 nM (2 ppb) Hg^{2+}. The assay is capable of discriminating Hg^{2+} from 15 other environmentally relevant metal ions. The method is attractive for potential point-of-use applications due to its high throughput, convenient readout, and portability.

Recently, a label-free silver-enhanced scanometric approach for in situ cell surface carbohydrate assay was designed by Ding et al. (33). Glyconanoparticles were prepared by a one-pot procedure. In the presence of lectin, using concanavalin A and mannose as models, the glyconanoparticles exhibited fast aggregation. The aggregation process could be inhibited by the specific recognition of lectin by the carbohydrate on the cell surface. The number of cell surface carbohydrate groups could be conveniently read out. The average number of mannose units on a single living intact bovine granulosa cell (BGC) cell was detected as $(4.5 \pm 0.4) \times 10^7$. The method takes advantage of nanoparticle-based recognition and aggregation-regulated signal amplification and avoids cell pretreatment and labeling processes.

CCD Camera Detection. After the process of silver enhancement, metallic silver is deposited onto the surface of gold nanoparticles, resulting in dark spots that can be optically measured using a CCD camera and quantified using computer software. A method for analyzing protein microarrays using a colorimetric gold nanoparticles probe coupled with silver enhancement was developed using CCD. Application of this method in an antigen microarray showed an identical result to fluorescent method (34). Immunoassays for bioanalytes such as biotin–peptide (35) and human IgG (36) detection using gold nanoparticles conjugated with antibodies have been developed. As low as 100 zmol of the biotin–peptide was detected after silver enhancement, and the linear working range was between 100 zmol and 100 fmol.

Visual Detection. An ultrasensitive colorimetric detection can result in visual detection without any instrument. As shown in Figure 3, visual detection of α-thrombin using

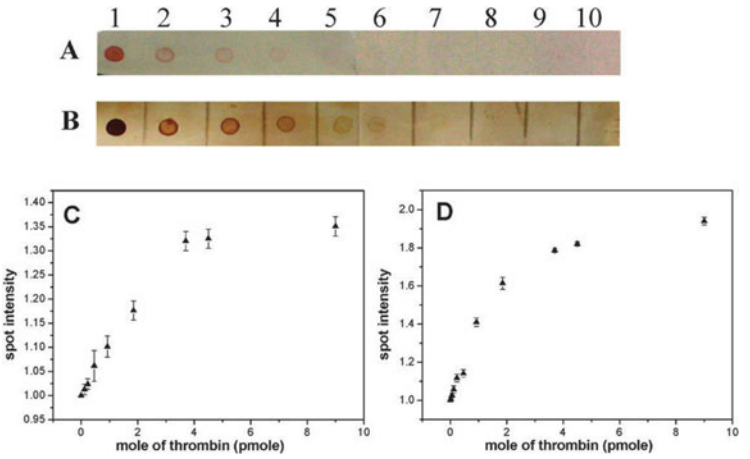

Figure 3. Dot images for colorimetric detection of a-thrombin (37) (A) without and (B) with silver enhancement for different levels of a-thrombin. The level of a-thrombin from 1 to 7 is 18.5, 3.7, 1.85, 0.925, 0.463, 0.230, and 0.115 pmol, respectively. Control experiments were conducted on 8, 9, 10 for 1.0 pmol of bovine serum albumin, b-thrombin, and g-thrombin, respectively. (C) Detection curve for a-thrombin corresponding to red dots in Figure 3A and (D) detection curve for a-thrombin corresponding to red dots (with silver enhancement) in Figure 3B. Reproduced from (37) with permission from the Royal Society of Chemistry. (Color figure available online.)

aptamer–Au nanoparticles conjugates based on a dot-blot array was developed by Wang et al. (37). The aptasensor has high sensitivity with the detection limit of 14 fmol with silver enhancement. Similar visual detection schemes were further applied to biomolecules such as adenosine (38) and antibodies (39).

Lateral flow immunoassays, also known as lateral flow immunochromatographic assays, are simple devices intended to detect the presence (or absence) of a target analyte in sample matrix without any specialized and costly equipment, especially with visual detection. Lateral flow immunoassay is a well-established and appropriate technology when applied to a wide variety of point-of-care or field use applications. The advantages of the lateral flow immunoassay system are well known, such as relatively low cost and minimal education required for users and regulators. However, their applications are often hindered by the lack of sensitivity. Silver enhancement has filled this gap. High-affinity anti-abrin-a monoclonal and polyclonal antibodies were used to develop a sandwich immunochromatographic assay and silver enhancement technology was used to further increase the sensitivity (40). A scheme of the immunochromatographic test device is shown in Figure 4. The visual detection limit was found to be 10 ng mL^{-1} before silver enhancement. The detection limit was 0.1 ng mL^{-1} for abrin-a, an increase in sensitivity of 100-fold, when silver enhancement technology was employed. To prevent the spread of highly pathogenic avian influenza virus among humans and to allow for timely medical intervention, a rapid and highly sensitive method is needed to detect and subtype the causative viruses. A silver-enhanced lateral flow immunoassay was developed for the highly sensitive and rapid diagnostic testing of hemagglutinin of H5 influenza viruses (41). The sensitivity of the test kit was increased 500 times by silver amplification. The sensitivity of the method was more than 10 times higher than that of conventional rapid influenza diagnostic tests that detect viral nucleoproteins.

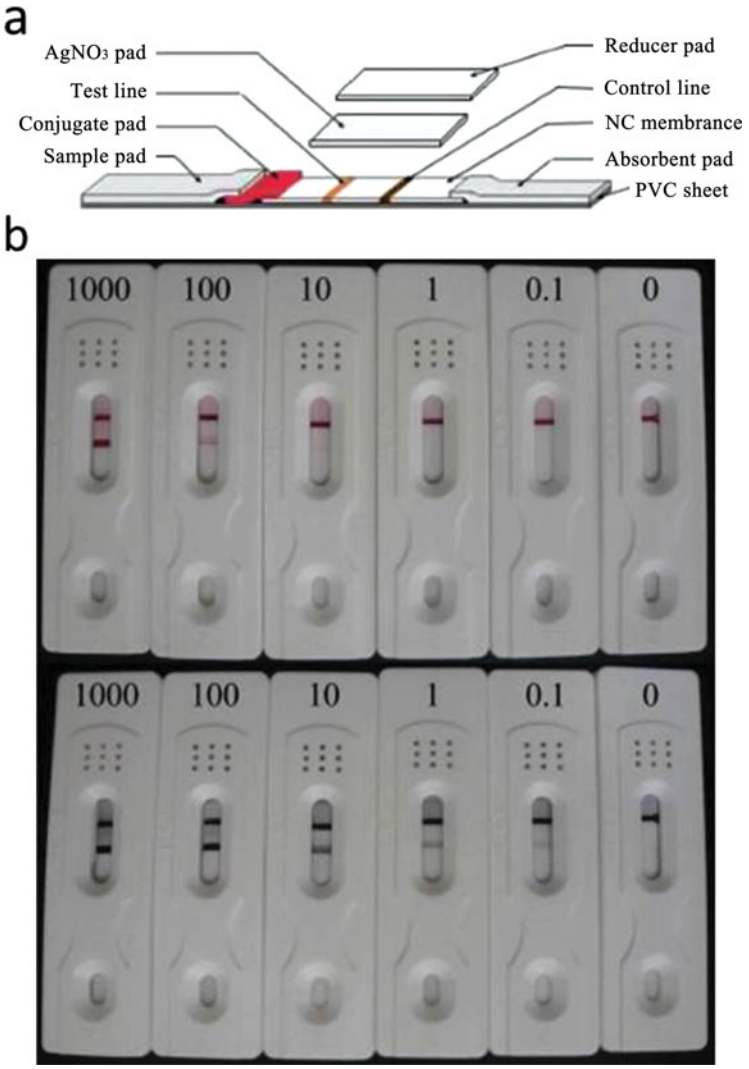

Figure 4. Schematic of the immunochromatographic test device (40). Detection limit of the test strip. Abrin-a standard solutions at concentrations of 0, 0.1, 1.0, 10, 100, and 1000 ng mL^{-1} were assayed. (a) The result of the assay before using silver enhancement technology and (b) amplified signal of the assay by application of silver enhancement technology. Reproduced from (40) with permission from Elsevier B.V. (Color figure available online.)

Electrical and Electrochemical Methods

Electrical Detection. Electrical methods for quantitative analysis are mainly based on physical measurements of capacitance or conductivity between two electrodes. They have the advantages of low cost, small instrument size, and high sensitivity, and it is especially suitable for microchip or microarray pattern detection. A DNA array detection method was reported in which the binding of oligonucleotides functionalized with gold nanoparticles led to conductivity changes associated with target-probe binding events (42). The binding

events localized gold nanoparticles in an electrode gap. The silver deposition facilitated by nanoparticles bridges the gap and led to readily measurable conductivity changes. Using this method, target DNA at concentrations as low as 500 fmol was detected with a point mutation selectivity factor of 100,000:1.

A silver-enhanced immunoassay method using microbeads and microbiochips was developed by Maeng et al. (43). Microbeads were used to filter and immobilize antibodies and an immunogold silver enhancement method was then used to amplify electrical signals that corresponded to the bound antibodies. The chip used for this system was composed of an inexpensive and biocompatible polydimethylsiloxane layer over a Pyrex glass substrate that contained a platinum microelectrode, which was used to detect the electrical signal. The microelectrode was fabricated on the substrate and a microchannel and pillar-type microfilter was formed in the polydimethylsiloxane (PDMS) layer. It was applied to detect α-fetoprotein (43) and protein A (44). A detection limit as low as 1 ng mL^{-1} was obtained.

Electrochemical Method. Electrochemical biosensors are often classified as amperometric, potentiometric, and conductometric biosensors. Anodic stripping voltammetry is often used in metal nanoparticle-based bioassays. In principle, it is a voltammetric method for quantitative determination of specific ionic species. The analyte of interest is deposited on the working electrode oxidized from the electrode during the stripping step. The current is measured during the stripping step. The oxidation of species is recorded as a peak in the current signal at the potential at which the species begins to be oxidized.

Analyzing sequence-specific DNA using gold nanoparticle DNA probes and subsequent signal amplification step by silver enhancement was developed by Cai et al. (45). The assay relies on the electrostatic adsorption of target oligonucleotides onto the sensing surface of the glassy carbon electrode and its hybridization to the gold nanoparticle–labeled oligonucleotides DNA probe. After silver deposition onto gold nanoparticles, binding events between the probe and target were monitored by the differential pulse voltammetry signal of the large number of silver atoms anchored on the hybrids at the electrode surface. The signal intensity difference allowed distinguishing between the match of two perfectly matched DNA strands and the mismatched base pair. Coupled with a silver signal amplification method, the sensitivity of this electrochemical DNA biosensor was increased by approximately two orders of magnitude and a detection limit of 50 pM of complementary oligonucleotides was obtained. A silver enhancement immunoassay was also developed with anodic stripping voltammetry detection. Similar assay schemes were applied for heterogeneous immunoassay of human IgG (46), *Schistosoma japonicum* antibody (14), carbohydrate-binding proteins (47), and specific DNA-binding protein (48). Graphene-based biosensors were constructed with good analytical performance (49, 50), due to the high DNA and protein loading ability of graphene and the distinct signal amplification by silver enhancement.

An electrochemical immunosensor array method was developed for simultaneous ultrasensitive measurement of tumor markers based on electrochemical stripping analysis of silver-enhanced gold nanoparticles (51). As shown in Figure 5, the immunosensor array was prepared by covalently immobilizing capture antibodies on chitosan-modified screen-printed carbon electrodes. The interference of dissolved oxygen was avoided because the detection was performed with positive stripping potential range. Using carcinoembryonic antigen and α-fetoprotein as model analytes, the proposed simultaneous immunoassay method showed wide linear ranges of three orders of magnitude with detection limits as low as 3.5 and 3.9 pg mL^{-1}, respectively.

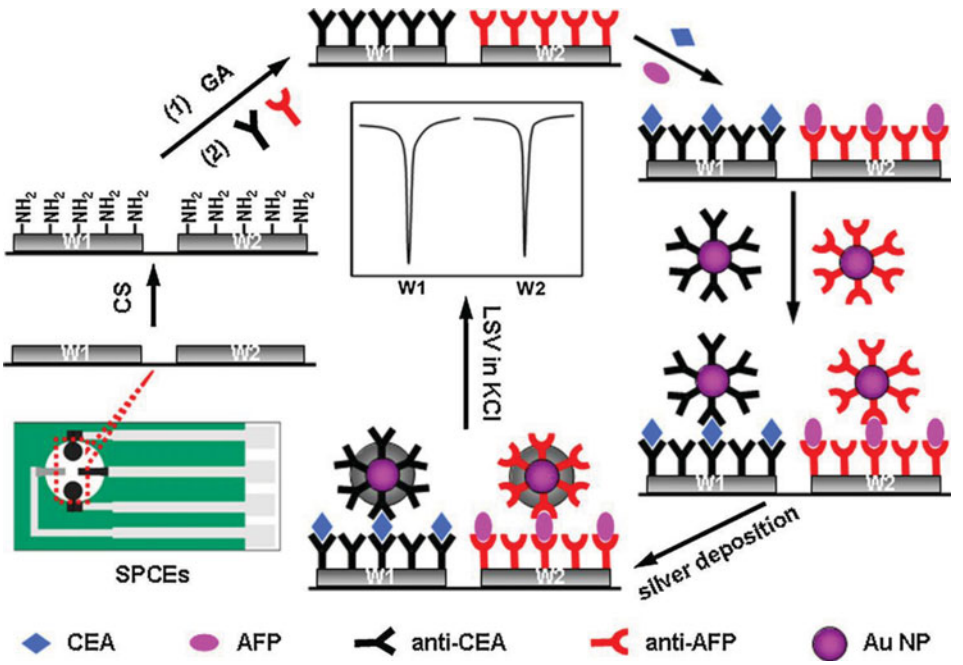

Figure 5. Schematic representation of preparation of immunosensor array and detection strategy by linear-sweep stripping voltammetric analysis of Ag nanoparticles catalytically deposited on the immunosensor surface by gold nanolabels (51). Reproduced from (51) with permission from Elsevier B.V. (Color figure available online.)

Other Methods

Gravimetric Detection. A silicon-based method was developed to couple silver enhancement with a flexural plate wave microgravimetric sensor, in which frequency shifts were due to the weight of the immunoglobulins (52). In addition to gravimetric sensing, the flexural plate wave device is capable of providing a source of ultrasonic agitation, which results in localized mixing near the active immunoglobulin binding area of the device. Later, a silver-enhanced microgravimetric quartz crystal microbalance sensor exhibited a two order of magnitude improvement in human IgG quantification (53).

Chemiluminescent Detection. A sensitive chemiluminescent immunoassay of human immunoglobulin that combined the inherent high sensitivity of chemiluminescent analysis with the signal amplification of silver precipitation on colloidal gold tags was developed by Li et al. (54). A large number of Ag^{+} ions were oxidatively released in HNO_3 solution from the silver metal anchored on the sandwich-type complexes and then the human IgG was indirectly determined by a sensitive combined chemiluminescent reaction of Ag^{+}-$K_2S_2O_8$-Mn^{2+}-H_3PO_4–luminol. The chemiluminescence intensity depended linearly on the logarithm of the concentration of human IgG over the range of 0.02–50 ng mL^{-1} and the LOD was 0.005 ng mL^{-1}.

Raman Spectroscopic Detection. Multiplexed detection of oligonucleotide targets has been performed with gold nanoparticle probes labeled with oligonucleotides and Raman-active dyes (55). The gold nanoparticles facilitate the formation of a silver coating, which acts as

a surface-enhanced Raman scattering promoter for the dye-labeled particles that have been captured by target molecules and an underlying chip in microarray format. The strategy provides the high sensitivity and high selectivity of grayscale scanometric detection but adds multiplexing capabilities because a very large number of probes can be designed based on the concept of using a Raman tag as a narrow-band spectroscopic fingerprint. Six dissimilar DNA targets with six Raman-labeled nanoparticle probes were distinguished, as well as two RNA targets with single nucleotide polymorphisms. The LOD was 20 fmol.

Resonance Light Scattering Detection. A silver-enhanced microarray for the detection of proteins and protein functionality (kinase activity) was developed based on resonance light scattering (56). Highly selective recognition of standard proteins (proteins A and G) down to 1 pg mL^{-1} for proteins in solution and 10 fg for proteins on the microarray spots was demonstrated. Enzyme activity of the kinase was detected with high specificity down to an LOD of 1 fg for kemptide on the microarray spots.

Inductively Coupled Plasma–Mass Spectrometric Detection. Inductively coupled plasma–mass spectrometry is the predominant and most sensitive commercial test for the determination of a wide range of metals and several nonmetals. The advantages of ICP-MS

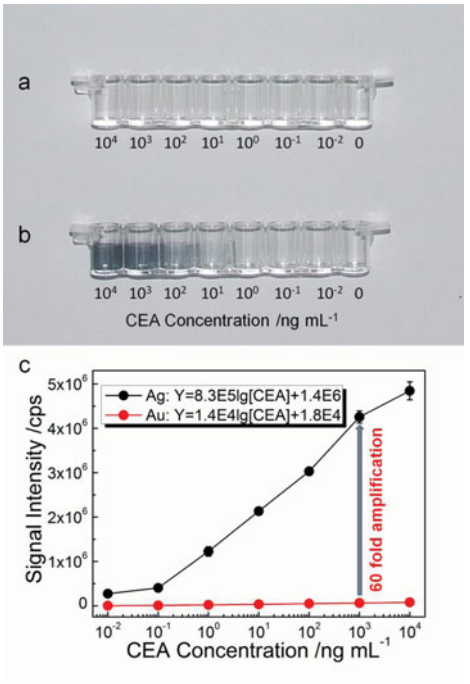

Figure 6. Relationship between the concentration of carcinoma embryonic antigen (CEA) and visible or ICP-MS signal (66). (a) Photograph of microtiter plate after immunogold-labeled sandwich immunoassay, (b) photograph of microtiter plate after immunogold-labeled sandwich immunoassay and silver amplification, and (c) ICP-MS Ag and Au signals after dissolving the metallic elements in the microtiter plate. Reproduced from (66) with permission from the American Chemical Society. (Color figure available online.)

include low detection limits, low matrix effects, large dynamic ranges, and high spectral resolution for elements and isotopes (57, 58). Therefore, element-tagged immunoassay with ICP-MS detection is an ideal detection technique for metal nanoparticle–based immunoassays (59–63). ICP-MS as a readout tool does not require nanoparticle reporters to possess optical, electric, electrochemical, magnetic, or any other special properties because atomic ions from the nanoparticle are directly detected (64). A distinguishing feature of ICP-MS is the great multiplexing potential for biological analytes endowed by the excellent element isotopic spectral resolution of the mass spectrometer. Another feature is that high sensitivity can be easily obtained by the use of the nanoparticle tag instead of metal ions, due to large quantities of detectable atoms in each nanoparticle tag (65, 66). As shown in Figure 6, a highly sensitive immunogold–silver-amplified ICP-MS immunoassay was developed (67) that combined the intrinsic high sensitivity of ICP-MS with the signal enhancement of immunogold–silver amplification. A carcinoembryonic antigen was chosen as the model analyte with a detection limit of 0.3 ng mL^{-1}.

Conclusion

The feasibility of highly sensitive bioassays based on the quantitative precipitation of silver onto immunogold tags has been demonstrated by various applications. The great signal enhancement by silver amplification has been successfully combined with some sensitive detectors. The silver amplification procedure is simple, low cost, and rapid. It shows great potential in qualitative biosensing as well as quantitative biosensing for numerous applications in sensitive immunoassays, DNA hybridization, and other biological analyses. Further research on silver enhancement in lateral flow immunoassays may result in fast and simple field detection of biomarkers with low abundance that were previously not detectable. ICP-MS is a powerful ultratrace element detection technique and thus has great potential for silver element detection after silver enhancement.

On the other hand, there are still several drawbacks and limitations of immunogold silver amplification methods, namely, (1) it is difficult to carry out simultaneous multiple analyte biosensing by immunogold–silver amplification, because silver shells covering gold nanoparticles without selectivity are not discriminable, (2) strict control of the reaction conditions such as reaction time is required for acceptable reproducibility, and (3) self-nucleation or nonspecific binding often occurs, resulting in a nonspecific signal. Continuing efforts to alleviate the interference of nonspecific binding are required, and further improvements in combined use of other signal amplification methods with silver enhancement for simultaneous multiple bioanalyte sensing would be welcome.

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