J. Anal. Test. (2017) 1:7
DOI 10.1007/s41664-017-0008-6

REVIEW

Signal Amplification for Highly Sensitive Immunosensing

Huangxian Ju[1]

Received: 11 January 2017 / Accepted: 17 January 2017 / Published online: 10 February 2017

Abstract To dissolve the bottleneck problem of life and biomedical science in detection of biomolecules with low abundance and acquisition of ultraweak biological signals, highly sensitive analytical methods coupling with the specificity of biological recognition events have been quickly developed by the exploring of signal amplification strategies. These strategies have extensively been introduced into the development of highly sensitive immunosensing methods by combining with highly specific immunological recognition. They can be divided into two groups. One group of strategies attempts to transfer the immunological recognition event into large number of reporter probes or signal probes for signal readout by employing nano/micro-materials as vehicles for multi-labeling and/or molecular biological amplification for increasing the abundance of the signal molecules. The other uses nanomaterials or enzyme mimics as catalytic tools/tags to obtain enhanced detection signal. This review focuses on the significant advances in design of signal amplification strategies for highly sensitive immunosensing.

Keywords Signal amplification · Immunosensing · Immunological recognition · Reporter probes · Nanoparticles · Enzyme mimics · Polymerase chain reaction · Rolling circle amplification · Hybridization chain reaction

1 Introduction

The detection of biomolecules with low abundance and the acquisition of ultraweak biological signals have been become a development bottleneck of life and biomedical science, because a few important biomolecules are sufficient to regulate the biological functions of cells and trigger disease processes, and numerous pathological biomarkers are present at very low levels during the early stages of the disease development. Developing highly sensitive analytical methods for detection of biomolecules coupling with the specificity of biological recognition events is in urgent need. Immunoassay combines the unique advantages of highly specific immunological recognition and convenient biosensing devices has shown its outstanding availability for detection of protein analytes. One of the major goals in developing novel immunosensing methods for the detection of protein biomarkers is to achieve high sensitivity. However, owing to the inherent background signals of various instrumental analytical technologies and the limitation of the classical analytical methodologies, improving the sensitivities via traditional physical methods or simple chemical and biocatalytic processes is far from meeting the practical demands. The progress in nanotechnology and biotechnology sets a convenient and promising way for design of signal amplification strategies [1–3], which leads to novel immunoassay methods with high sensitivity.

The signal amplification strategies for highly sensitive immunosensing of proteins mainly include two groups. One group of strategy attempts to transfer protein target-binding events into large number of reporter molecules or signal molecules for signal readout. These strategies commonly employ nano/micro-materials as vehicles for multi-labeling or molecular biological amplification for

---

✉ Huangxian Ju
hxju@nju.edu.cn

[1] State Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing 210023, China

increasing the amount of signal molecules. Another group uses nano/micro-materials or enzyme mimics as catalytic tools to obtain enhanced detection signal. This review focuses on the recent development of highly sensitive immunosensing methods with different signal amplification strategies.

2 Nanomaterials as Carriers for Multi-Labeling Immunosensing

Immunoassay is usually performed by competitive or sandwich formats. Both of them utilize a label or tag as the signal probe for quantifying the antigen–antibody reaction (Fig. 1). In traditional immunoassay, the labeled antibody or antigen, which usually is linked with a label molecule, such as enzyme, illuminant dye and radioactive element at a ratio of 1:1, is employed to specifically recognize the antigen or antibody and indicate the detection signal [4]. There is no doubt that the low ratio of signal label to antibody or antigen results in limited detectable signal and relatively low sensitivity of these traditional immunoassays. Thus, the first consideration for amplifying the detection signal is to use more label molecules for one immunological recognition event. In recent years, great efforts have been made to amplify the signal output by increasing the ratio of signal tag to specific recognition ligand using nanomaterials as carriers [5, 6] or signal tags, or molecular biological technology [7, 8] to introduce more reporter molecules. Due to the high surface-to-volume ratio of nanomaterials, it is an efficient method to use them as the carriers labeling numerous reporter molecules by immobilizing enzymes or nanoparticle on their surface. The large number of reporter molecules on the surface produce an enhanced signal for the immunoassay of the target molecules.

2.1 Nanoparticle-Functionalized Nanoprobes for Signal Amplification

Nanoparticles generally exhibit special electrochemical or optical activity and, thus, they are popularly employed for signal tracing of immunoassay. For example, quantum dots (QDs), as semiconductors, can be excited by chemical and electrochemical reactions. The light emission of QDs controlled by the applied electrode potential enables them to be applied as excellent ECL labels for bioassays [9]. The use of CdTe QDs as the luminophor and $K_2S_2O_8$ as the coreactant is the most popular system in sandwich-enhanced ECL immunoassays. A multi-labeling strategy for sensitive detection of tumor necrosis factor-alpha (TNF-α) has been reported using QDs–polymer-functionalized silica nanosphere as the label [10]. The nanosphere leads to an increase of CdTe QD loading per immunoassay event and, thus, increases the signals of electrochemiluminescence (ECL) and square-wave voltammetry (SWV) measurements by 10.0- and 5.5-folds, respectively, in comparison with the unamplified method. A multi-

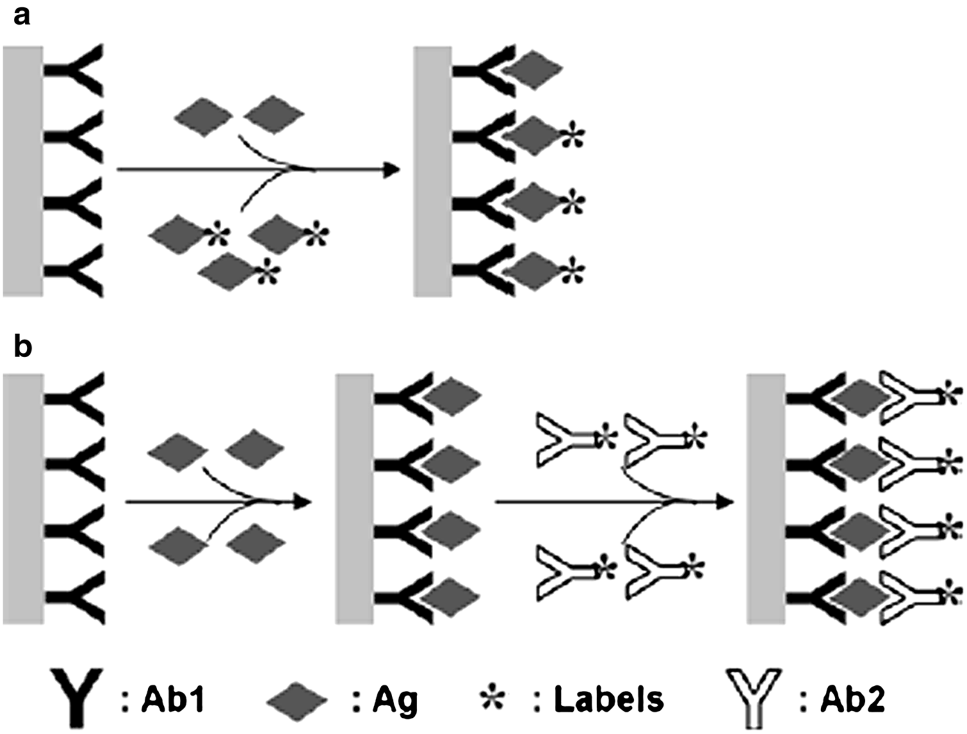


Fig. 1 Schematic illustration of competitive immunoassay (**a**) and sandwich immunoassay (**b**)

labeling strategy based on the combination of tyramide signal amplification (TSA) and polymerization-based amplification (PBA) has also been proposed [11]. The surface-initiated atom transfer radical polymerization of glycidyl methacrylate (GMA) is triggered by the initiator-coupled antibody immobilized on the surface of biosensor via traditional sandwich immunoreactions (Fig. 2). Growth of long-chain polymeric materials provided numerous epoxy groups for subsequent coupling of horseradish peroxidase (HRP), which in turn significantly increased the loading of QDs labeled tyramide in the presence of hydrogen peroxide due to the TSA mechanism. As a result, the ECL and SWV measurements show 9.4- and 10.5-fold increase in detection signal in comparison with the unamplified method, respectively.

2.2 Enzyme-Functionalized Nanoprobes for Signal Amplification

Due to the highly efficient bioactivity of enzymatic reactions, enzyme labels are frequently used to functionalize various nanomaterials for the preparation of nanoprobes in immunoassays. The unique properties of the enzyme-functionalized nanoprobes enable the development of a large variety of signal transduction strategies for ultrasensitive immunosensing of protein biomarkers. For example, Rusling's group reported a multi-HRP functionalized multiwall carbon nanotubes (MWCNTs)-based nanoprobe for the highly sensitive detection of a cancer biomarker [12]. The nanoprobe was prepared through covalently linking high ratio of HRP labels and secondary antibodies onto the surface of the carboxylated MWCNT nanocarrier. When it was used for the signal tracing of sandwich immunoreaction at the immunosensor, greatly enhanced sensitivity was achieved based on the multi-label signal amplification (about 90 catalytic labels per binding event on the immunosensor surface) and the enzyme-catalytic amperometric response from the HRP labels (Fig. 3). This approach provided a low detection limit of 4 pg/mL (100 amol/mL) for PSA measurement in serum and tissue lysates.

The large specific surface area of graphene enables it to act as an ideal nanocarrier for nanoprobe preparation. A HRP-functionalized graphene oxide nanoprobe has been prepared to develop an impedimetric immunosensors by the catalyzed oxidation of 4-chloro-1-naphthol substrate to induce the production an insoluble precipitate [13]. Upon sandwich immunoreaction followed by enzymatic reaction induced by the quantitatively captured nanoprobe, a layer of insoluble benzo-4-chlorohexadienone product is deposited on the electrode surface, thus hindering the electron transfer process of the redox probes between the base electrode and the solution and enhancing the sensitivity of the impedimetric immunosensors. Lai et al. [14] employed this enzymatic reaction to develop an ultrasensitive electrochemical immunosensor based on the amplified inhibition of the electrochemical signal of ferrocene indicator immobilized on the immunosensor.

The gold nanostructures at various size/shape (mostly Au NPs) have high specific surface area, high surface energy, high conductivity and offer numerous adsorption sites to the biomolecules such as antibodies, enzymes and proteins which make them an ideal choice for the biosensor and nanoprobe construction. Ju's group [15] designed a

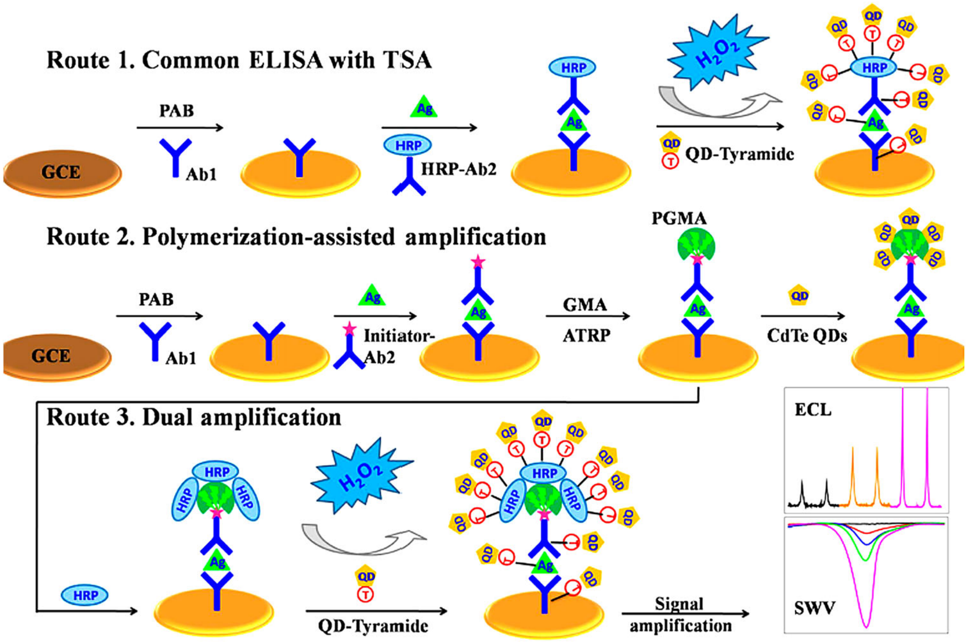


Fig. 2 Schematic representation of typical TSA based on common ELISA using QD–Tyramide conjugates as labels (*Route 1*), the polymerization-assisted amplification in sandwich immunoassay via surface-initiated ATRP and subsequent direct binding of CdTe QDs (*Route 2*), and the sandwich immunoassay using QD–Tyramide conjugates as labels via surface-initiated ATRP and TSA (*Route 3*)

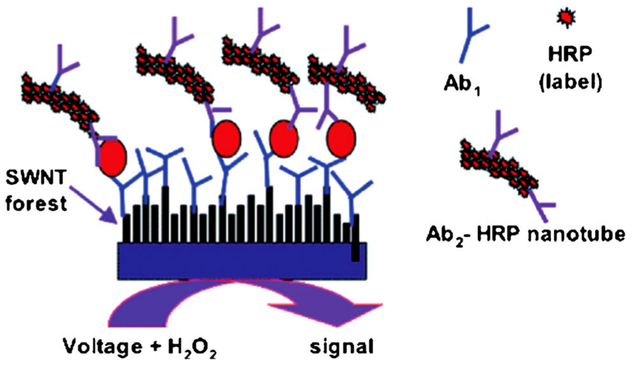


Fig. 3 Illustration of detection principles of the immunosensor after treating with HRP-SWCNT nanoprobe to obtain amplification by providing numerous enzyme labels per binding event

glucose oxidase (GOx) functionalized CNT/Au NPs nanoprobe through a layer-by-layer assembly method and used it to develop an ultrasensitive multiplexed electrochemical immunosensing method by combination with a disposable immunosensor array (Fig. 4). The immunosensor array was constructed through the stepwise assembly of colloidal Prussian blue (PB), Au NPs and captured antibodies on screen-printed carbon electrodes. The enzymatic reaction of the nanoprobe provided sensitive electrochemical signal tracing of the immunosensors. The colloidal PB acted as a mediator to catalyze the reduction of H_2O_2 produced in the GOx enzymatic reaction. Both the high-content GOx and CNT/Au NPs in the nanocomposite tracer amplified the detectable signal of the immunosensor. Using carcinoembryonic antigen and α-fetoprotein as model analytes, the simultaneous multiplexed immunoassay method using the immunosensor array and the designed tracer showed linear ranges of three orders of magnitude with the detection limits down to 1.4 and 2.2 pg mL^{-1}, respectively. The assay results of serum samples with the proposed method were in an acceptable agreement with the reference values. The dual-signal amplification of glucose oxidase-functionalized nanocomposites provided a promising ultrasensitive simultaneous multiplexed immunoassay approach for clinical applications.

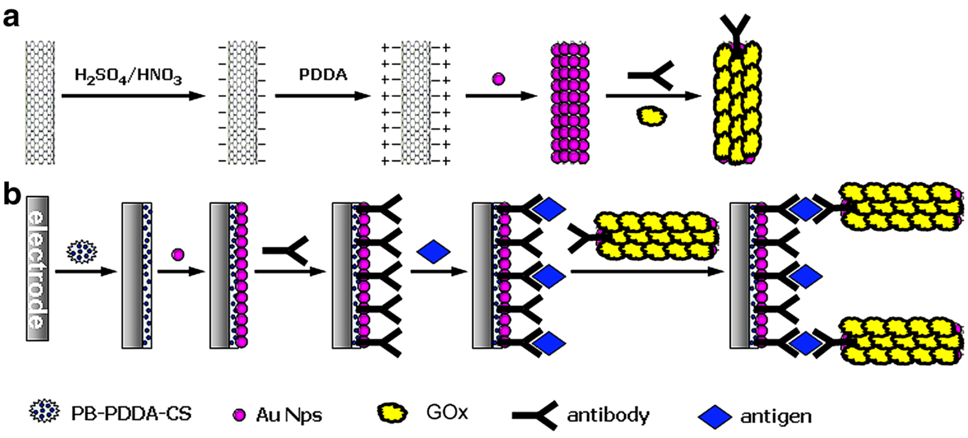
3 Nanoparticles as Signal Labels for Signal Amplification

Nanoparticles, such as semiconductor nanoparticles and metal oxide nanostructures, have been considered as potential signal labels to stimulate the development of signal amplification strategy for immunosensong [16, 17]. The enormous signal enhancement associated with the use of nano labels provides the basis for ultrasensitive immunosensor and immunoassays with different measurement techniques [18].

3.1 Electrochemical Stripping Analysis of Nano Labels for Signal Amplification

Electrochemical immunosensors offer high sensitivity, uncomplicated instrumentation and capability in protein determination. Different nano labels have been designed for amperometric [19], potentiometric [20], impedimetric [21] and capacitometric analysis [22]. The electrochemical stripping analysis of Au NPs or silver nanoparticles (Ag NPs) is an extensively used signal amplification technology due to the high amount of metal ion in nanoparticle labeled to antibody. Furthermore, these nanoparticles can induce the deposition Ag NPs for further amplifying the electrochemical stripping signals. Ju' group [23] reported a simple, sensitive and low-cost multiplexed immunoassay by combining a disposable chip with Au NP as an electrochemical label (Fig. 5). The immunosensors array as the disposable chip was firstly prepared by immobilizing capture antibodies on different screen-printed carbon working electrodes by passive adsorption, respectively. With a sandwich mode, the analytes were then bound to the corresponding capture antibodies for further capture of the

Fig. 4 Schematic representation of **a** preparation procedure of GOx-Au Nps/CNTs nanoprobe and **b** preparation of immunosensors and sandwich electrochemical immunoassay



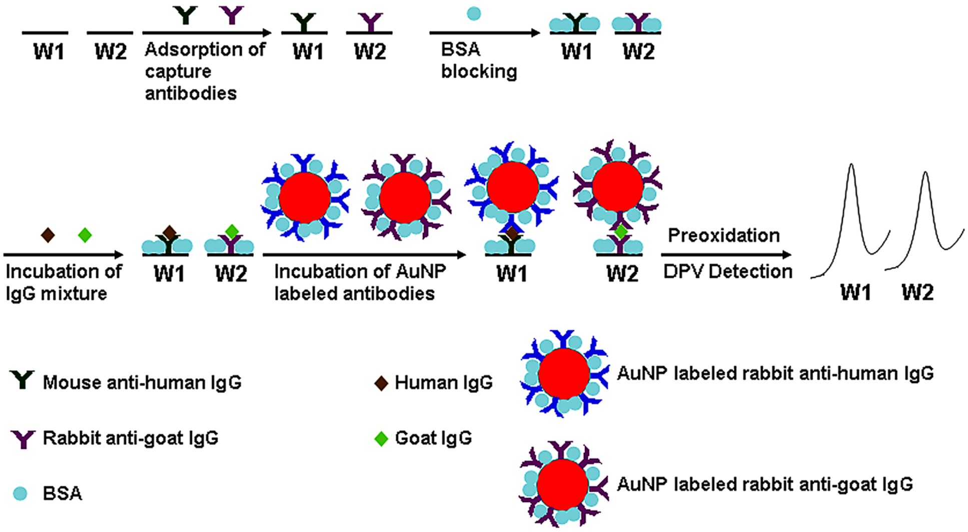


Fig. 5 Schematic representation of preparation of immunosensors array and analytical procedure for simultaneous detection of human IgG and goat IgG

gold nanoparticle labeled antibodies. Au NP labels were finally electrooxidized in 0.1 M HCl to produce AuCl$_4^-$ for differential pulse voltammetric detection. Using human IgG and goat IgG as model targets, under optimal conditions this method achieved linear ranges from 5.0 to 500 and 5.0 to 400 ng mL^{-1} with limits of detection of 1.1 and 1.6 ng mL^{-1}, respectively. Lai et al. [24] employed the enzymatically catalytic deposition of Au NPs to develop an ultrasensitive electrochemical immunosensing method by combination with gold stripping analysis at a CNT-based immunosensor. The gold nanoprobe was prepared by loading signal antibody and high-content GOx on the nanocarrier of gold nanorod. After sandwich immunoreaction, the GOx-Au NR nanoprobe quantitatively captured onto the immunosensor surface induced the deposition of Au NPs via the enzymatically catalytic reaction. Based on the electrochemical stripping analysis of the Au NR nanocarriers and the enzymatically produced Au NPs, sensitive electrochemical signal was obtained for the immunoassay. Both the GOx-induced deposition of Au NPs by the nanoprobe and the sensitive electrochemical stripping analysis on the CNTs-based sensing surface greatly amplified the signal response, leading to the ultrasensitive measurement of CEA in a wide linear range from 0.01 to 100 ng mL^{-1} with a detection limit down to 4.2 pg mL^{-1}.

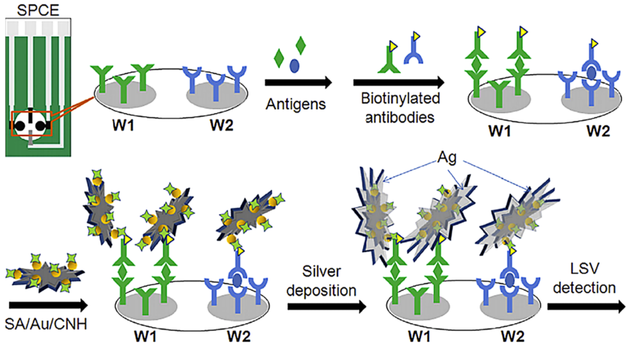


Fig. 6 Schematic representation of sandwich-type immunoassay procedure

Due to the lower oxidation potential and facile stripping conditions, Ag NPs are often functionalized with the nanocarriers such as CNT [25], graphene [26, 27] and carbon nanosphere [28] for highly sensitive immunoassay by direct electrochemical stripping detection. Zhao et al. [29] developed an ultrasensitive multiplexed immunoassay method via using streptavidin/nanogold/carbon nanohorn (SA/Au/CNH) as a novel signal tag to induce silver enhancement for signal amplification (Fig. 6). Carbon nanohorn has been used as an excellent nanocarrier to load high amount of gold nanoparticles based on the unique features of large surface area, plentiful inner nanospaces, highly defective horns, good electrical conductivity. To realize multiplexed detection of a-fetoprotein and CEA, a nanocomposite was prepared by loading numerous streptavidin modified Au NPs on carbon nanohorn as signal tag to recognize biotinylated signal antibody, and then the SA/Au/CNH tag was captured on the immunoconjugates immobilized in disposable screen-printed electrodes to induce silver deposition and amplify the electrochemical stripping signals. Through sandwich-type immunoreaction and biotin–streptavidin affinity reaction amplifying sensing signals, the proposed immunosensor showed wide linear ranges with the detection limits down to 0.024 and 0.032 pg mL^{-1}, respectively.

Similarly, Ju's group [30] reported an ultrasensitive multiplexed immunosensing method developed by combination with an ALP-labeled antibody functionalized Au NP probe and the enzyme-Au NP catalyzed deposition of Ag NPs at a disposable immunosensor array (Fig. 7). After

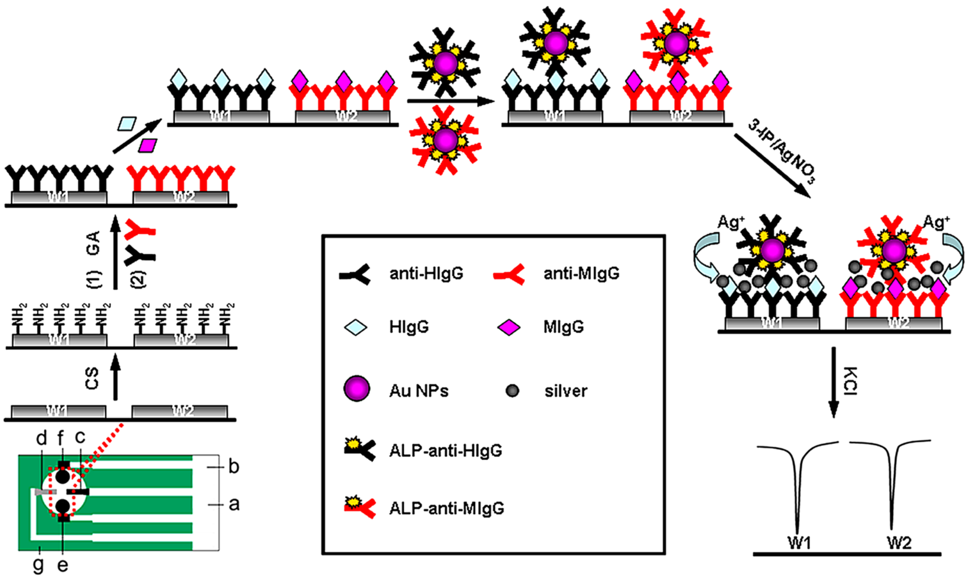


Fig. 7 Schematic representation of preparation of immunosensor array and detection strategy by sandwich immunoassay and linear sweep voltammetric stripping analysis of enzymatically deposited Ag NPs

sandwich immunoreaction at the immunosensor, the Au NP/ALP nanoprobes were quantitatively captured onto the immunosensing surface, and the enzymatic reaction of the nanoprobes induced the catalytic deposition of Ag NPs. The silver deposition process was catalyzed by both ALP and Au NPs, which amplified the detection signal. Based on the anodic stripping analysis of Ag NPs in KCl solution, this multiplexed immunosensor showed excellent analytical performance for simultaneous ultrasensitive measurement of the model analytes of human IgG and mouse IgG with wide linear ranges over four orders of magnitude with the detection limits down to 4.8 and 6.1 pg mL^{-1}, respectively.

Lin et al. [31] further utilized nanogold functionalized mesoporous carbon foam (Au/MCF) as signal label for sensitive electrochemical immunosensing of biomarker. The high loading of nanogold on MCF caused a C–Au synergistic silver enhancement and hence greatly amplified the detection signal and improved the detection sensitivity. Through a sandwich-type immunoreaction, Au/MCF tags were immobilized on an electrochemically reduced grapheneoxide/chitosan film modified glassy carbon electrode to induce a silver deposition process monitored by the electrochemical stripping signal. The Au/MCF-mediated silver enhancement along with the graphene-promoted electron transfer led to high detection sensitivity of CEA with a detection limit down to 0.024 pg mL^{-1}.

The silver deposition reaction can also be catalyzed by Ag NPs. Ju's group [25] designed a streptavidin-functionalized Ag NPs-enriched CNT/Ag NP as trace tag for the ultrasensitive multiplexed measurements of tumor markers using a disposable immunosensor array (Fig. 8). Due to the inherent reduction property of carboxylated CNT, Ag NPs were firstly in situ deposited on this support from silver ions in solution without the addition of any other reduction agent, which was further used for linking antibody through the streptavidin assembly. Through a sandwich-type immunoreaction at the immunosensor, the high-content Ag NPs were captured onto the immunosensor surface to further induce the silver deposition for amplifying the detection signal greatly. Based on the electrochemical stripping detection of the Ag NPs at the immunosensor, this method showed ultrahigh sensitivity with wide detection ranges and the detection limits down to 0.093 and 0.061 pg mL^{-1} for simultaneous electrochemical measurement of CEA and AFP.

The stripping voltammetric detection of Au NPs for immunosensing could also be achieved with gold nanorods (AuNRs) superstructure [32]. The amplification pathway firstly used a thio-β-cyclodextrin (SH-β-CD) functionalized gold nanoparticles to label signal antibody, and then in situ assembled multi-layer SH-β-CD end-functionalized AuNRs to sandwich immunocomplex on immunosensor surface using 4,4,4,4-(21H, 23H-porphine-5,10,15,20-tetrayl) tetrakis (benzoic acid) as a bridge to achieve simple and convenient host–guest reaction (Fig. 9). The built end-to-end AuNRs superstructure showed excellent performance for the signal amplification in connection with the electrochemical biosensing by preoxidation and then voltammetric analysis of gold element. Using α-fetoprotein as an analyte, the immunosensor was constructed by covalently binding capture antibody to chitosan–carbon nanotubes-poly(diallyldimethylammonium chloride) modified electrode. The superstructure rich in AuNRs brought an enhanced detection sensitivity of protein, which could detect α-fetoprotein in a linear range of 0.5 pg mL^{-1} to 0.5 ng mL^{-1} with a detection limit down to 0.032 pg mL^{-1}.

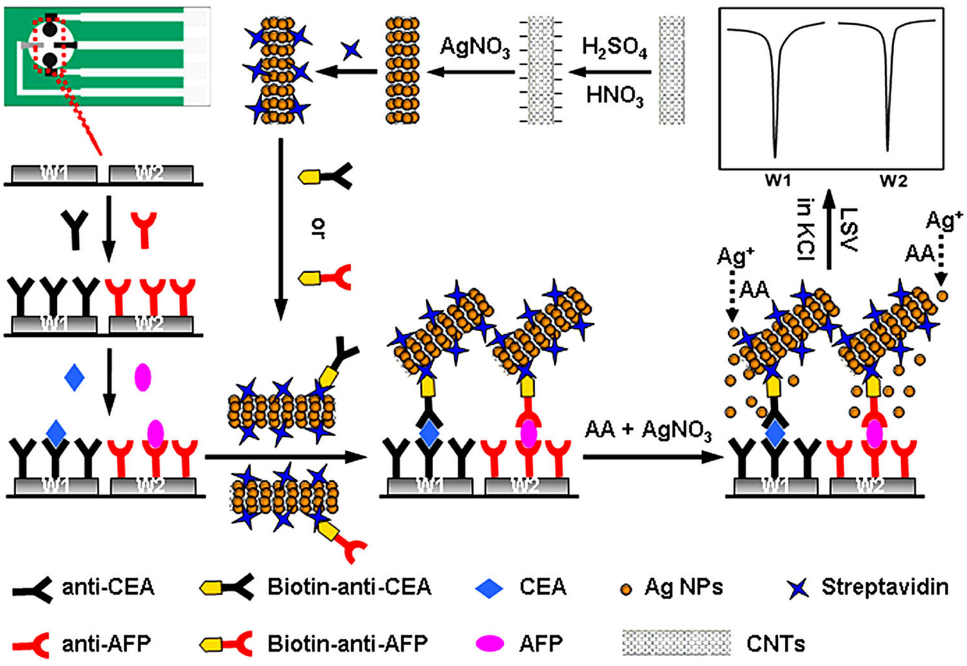


Fig. 8 Schematic representation of preparation of immunosensor array and trace tag, and detection strategy by linear sweep stripping voltammetric analysis of Ag NPs on the immunosensor surface

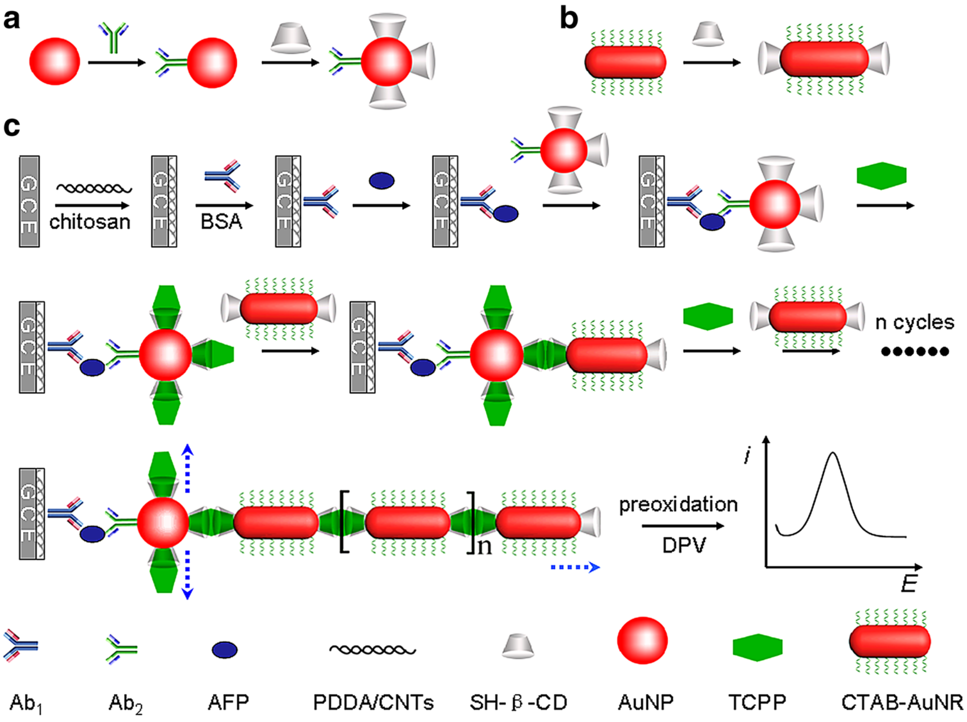


Fig. 9 Schematic representation of the preparation of **a** CD-Ab_2/AuNPs and **b** CD/AuNRs Tags; **c** immunosensor fabrication and sandwich-type immunoassay with AuNRs superstructure based signal amplification

3.2 Signal Amplification in Optical-based Technologies

Optical-based technologies, such as chemiluminescent (CL), ECL, fluorescence, surface-plasmon resonance (SPR) and Raman scattering technologies, are the most popular and convenient tools for immunoassay due to the advantages of applying visible radiation, nondestructive operation mode and the rapid signal generation and reading [33, 34]. Shourian et al. [35] designed a multi-functionalized AuNPs as signal label for highly sensitive immunoassay of hepatitis B surface antigen (HBsAg). The AuNPs were functionalized by bearing biotin and luminol molecules, and then the multi-functionalized AuNPs coupled with streptavidin modified secondary antibody which was immobilized in polystyrene well via the primary monoclonal antibodies bound with HBsAg. The CL signal was produced by oxidation of luminol molecules in the presence of $HAuCl_4$ as catalyst and H_2O_2 as oxidant. The immunosensor showed about 40 times lower detection limit toward HBsAg relative to previous work [36]. In such a case, the nanoparticles act as both a solid carrier to load a large number of reporters/markers and labels to amplify the CL signal for the protein detection.

Based on mesoporous SiO_2-coated CNTs–QDs nanocomposites and ECL energy transfer (ECL-ET) technology, Zhu's group [37] fabricated a versatile biosensor via employing the energy tunable CdSeTe/CdS/ZnS double shell QDs as donor and gold nanorods (GNRs) as acceptor (Fig. 10). Surprisingly, they proposed a microwave-assisted production of high quality NIR-emitting CdSeTe/CdS/ZnS QDs with successive epitaxial CdS and ZnS double shells. The double CdSeTe/CdS/ZnS QDs manifested perfect energy match with gold nanorods (GNRs) as the donor–quencher pair. Due to the mCNTs–QDs and ECL-ET technology twofold signal amplification, this approach provided a sensitive response to CEA in a wide range from 0.001 to 200 pg mL^{-1} with a detection limit of 0.5 fg mL^{-1}.

4 Enzyme Mimics for Signal Amplification

Artificial mimicking enzymes possessing high catalytic activity and distinct substrate selectivity are highly desired for developing new catalytic reactions and bioanalysis systems due to their simpler syntheses and preparation, better stability, and easier modification than natural enzymes [38]. Therefore, lots of researches have focused on the development of enzyme mimetics-based signal amplification for immunosensing using coordination compounds with peroxidase activity, such as hemin and porphyrin, and catalytic/electroactive nanoparticles, especially metal and semiconductor nanoparticles as labels [39–43].

As the catalytic centers of many enzymes, hemin has been widely used for the development of artificial enzymes. It can conjugate to G-quadruplex to form DNAzyme with peroxidase-mimicking activity [44, 45]. Ju's group [46] designed a multilayer G-quadruplex/hemin DNAzyme wrapped gold nanoparticle (M-DNAzyme/AuNP) tag for ultrasensitive CL image analysis of proteins (Fig. 11). The M-DNAzyme/AuNP tag was prepared by assembling high ratio of alkylthiol-capped signal DNA containing multiple G-quadruplex sequences to biotinylated DNA on AuNPs and then reacting with hemin to form multilayer hemin/G-quadruplex DNAzyme units. It was bound to the biotinylated secondary antibody of sandwich immunocomplex by biotin–streptavidin conjugation to catalyze a CL reaction on a protein array, which produced strong CL emission. By combining with a disposable protein array, an ultrasensitive and high-throughput multiplex CL immunoassay method was proposed for simultaneous detection of four cancer biomarkers, including fetoprotein, human chorionic gonadotrophin-β, carcinoma antigen 125, and CEA, with the limits of detection of 2.7×10^{-5} ng mL^{-1}, 1.1×10^{-5} IU mL^{-1}, 1.7×10^{-5} U mL^{-1}, 2.0×10^{-5} ng mL^{-1}, respectively.

Xu et al. [47] designed a mimicking enzyme with peroxidase-like activity by loading MnTMPyP in the dsDNA scaffold. This complex possessed high catalytic activity and much quicker catalytic kinetics and better stability with exposure to light irradiation and high temperature than both

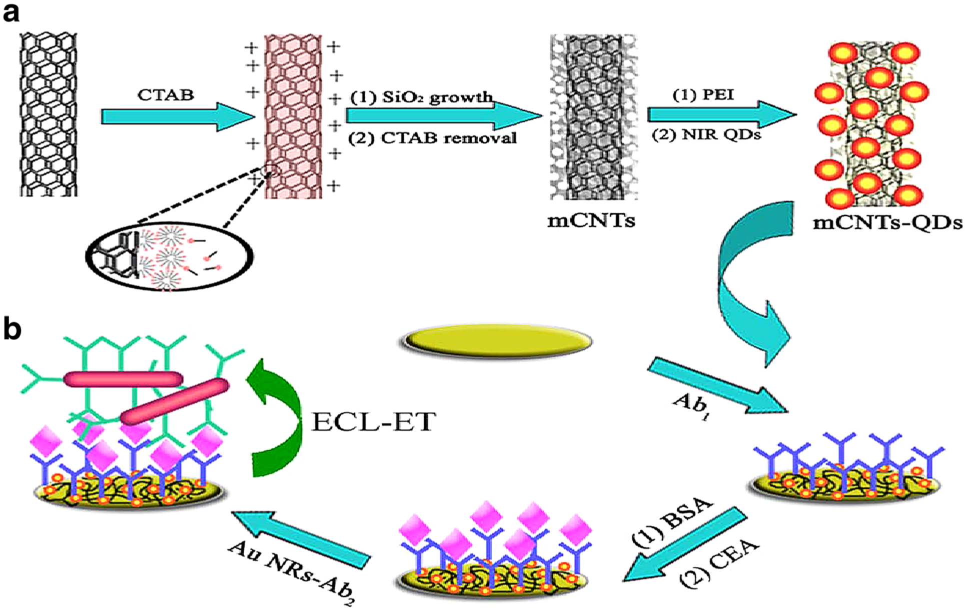


Fig. 10 Construction of the ECL immunosensor. **a** Schematic representation of preparation procedure for mCNTs–QDs composites. **b** Schematic illustration of the stepwise immunosensor fabrication process

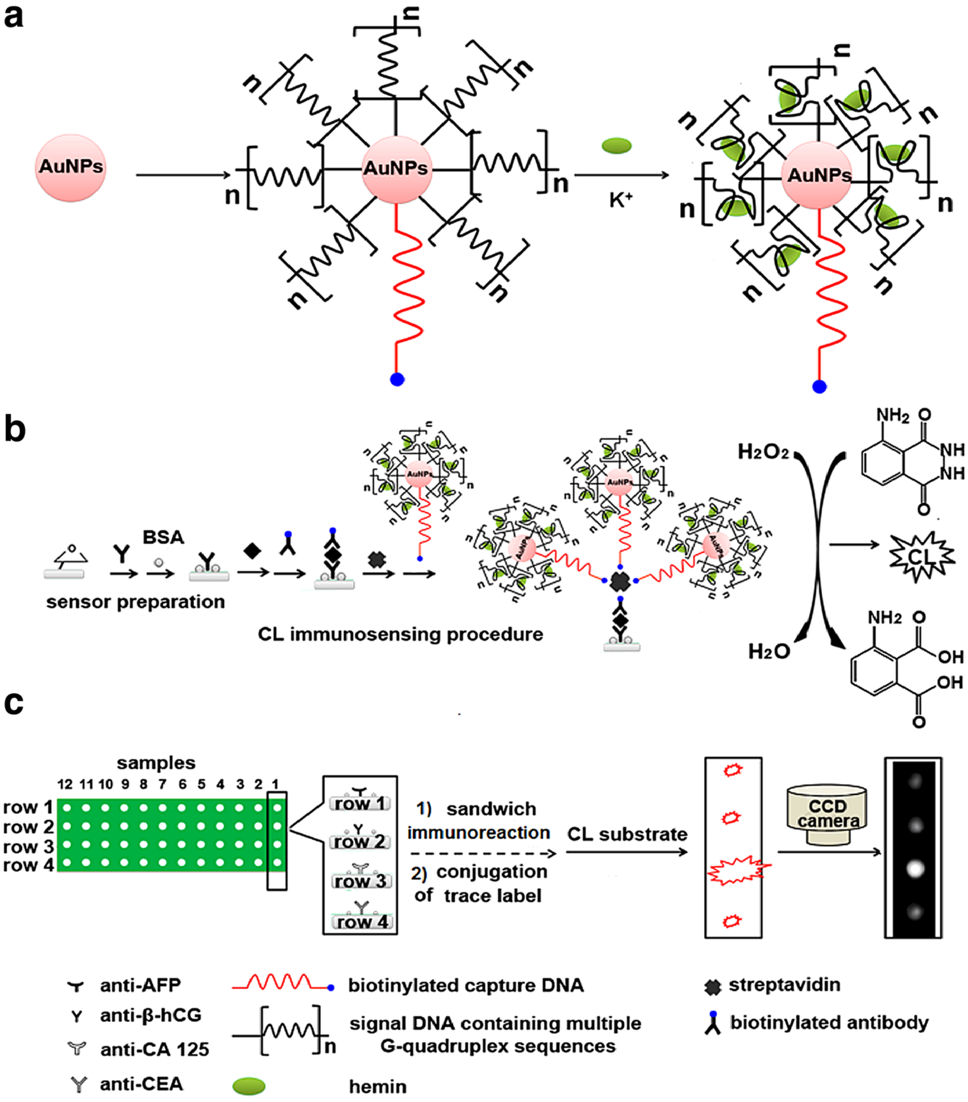


Fig. 11 Schematic diagrams of **a** preparation of M-DNAzyme/AuNP, **b** sensor preparation and CL immunosensing procedure, and **c** multiplex CL imaging immunoassay of four tumor markers using an immunosensor array

horseradish peroxidase and hemin/G-quadruplex DNAzyme. By combining with an isothermal hybridization chain reaction (HCR) and in situ formation of MnTMPyP–dsDNA, a highly efficient chemiluminescent (CL) immunosensing method was proposed (Fig. 12). After a sandwich immunoreaction, a biotinylated DNA strand, which was bound to biotinylated signal antibody by streptavidin, triggered the HCR and growth of MnTMPyP–dsDNA on the immunocomplex. The in situ, HCR-assisted enzyme formation brought numerous enzymatic catalytic centers, MnTMPyP, on the immunocomplex, resulting in significant CL signal amplification and highly sensitive CL detection.

Similarly, a G-quadruplex/hemin-Pt NP was used as enzyme mimetics probe for achieving ultrasensitive photoelectrochemical (PEC) immunoassay [48]. The G-quadruplex/hemin-Pt NP probe could catalyze the oxidation of hydroquinone (HQ) using H_2O_2 as an oxidant due

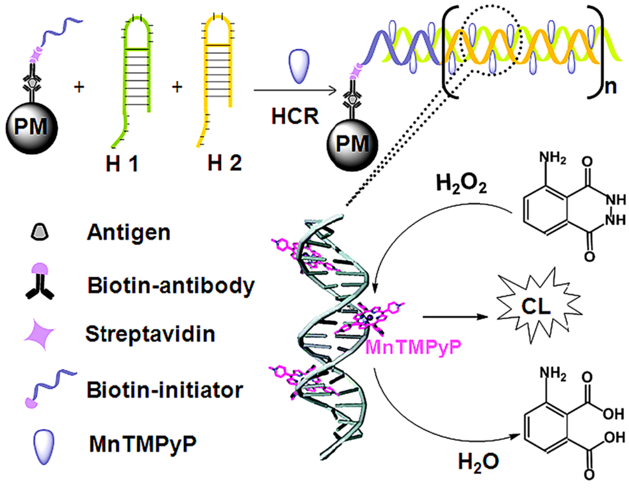


Fig. 12 Schematic illustration of amplifying synthesis of MnTMPyP-dsDNA enzyme mimic as label for CL biosensing Reprinted with permission from [69]

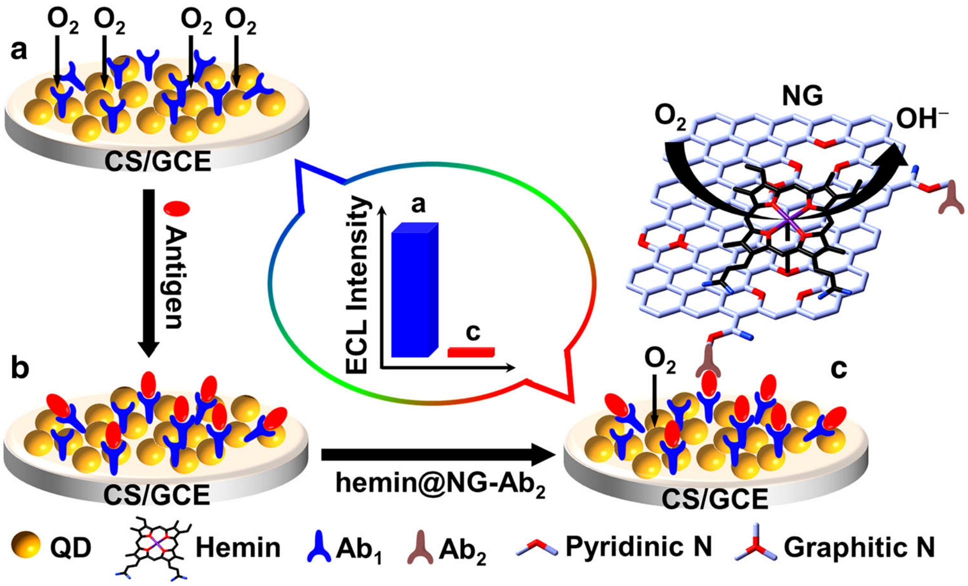
to its peroxidase-mimicking activity. The oxidation product of HQ, a polymer with abundant electron accepting groups, could efficiently inhibit the photocurrent of CdS QDs through photoinduced electron transfer and steric hindrances effect, resulting in a highly sensitive PEC immunosensor.

Ju's group [49] also prepared a hemin functionalized graphene sheet via the noncovalent assembly of hemin on nitrogen-doped grapheme (NG). The hemin@NG could act as an oxygen reduction catalyst to produce sensitive ECL quenching of quantum dots (QDs) due to the annihilation of dissolved oxygen, the ECL coreactant, by its electrocatalytic reduction. The hemin@NG was proved to produce higher catalytic efficiency than hemin@GO to quench the cathodic ECL emission of QDs. With a sandwich immunoassay format using an immunosensor constructed by immobilization of bidentate-chelated CdTe QDs and capture antibody, the hemin@NG as a tracing tag showed higher sensitivity (Fig. 13). With the use of CEA as a model, the proposed ultrasensitive immunoassay method could detect the protein biomarker down to the subpicomolar level with a detection range over 5 orders of magnitude.

Nanoparticles especially metal and semiconductor nanoparticles with enzyme-like activities can be used directly as electroactive labels for the electrochemical amplification detection of proteins. Fe_3O_4 NPs and Fe_3O_4 NPs-based hybrid materials can exhibit enhanced peroxidase-like activity [50–52]. Wang et al. [53] synthesized Au NPs doped Fe_3O_4 (Au@Fe_3O_4) NPs by a facile one-step solvothermal method. The peroxidase-like activity of Au@Fe_3O_4NPs was effectively enhanced due to the synergistic effect between the Fe_3O_4NPs and Au NPs. On this basis, an efficient colorimetric aptasensor was developed for the detection of ochratoxin A using the intrinsic dual functionality of the Au@Fe_3O_4 NPs as signal indicator. Wei et al. [54] developed an enzyme mimic immuno-label by loading Fe_3O_4NPs into a polymeric vesicle, poly (ethylene glycol) epoly (lactic acid) (PEG-PLA), followed by conjugating secondary antibody (Ab2) onto the vesicles surface. The resulting Ab_2@-PEG@PLA@Fe_3O_4 demonstrated high catalytic activity towards H_2O_2, and the sensitivity of the sandwich-type immunosensor using this label for prostate PSA detection increased greatly.

Metallic alloy nanomaterials with enzyme-like activities have also attracted great attentions due to their better catalytic properties than monometallic counterparts, such as Pt–Au nanoparticles [55], Au–Pd nanostructures [56] and AuPd bimetallic nanoprobe [57]. These metallic alloy nanomaterials with excellent electrocatalytic performance, large surface area and favorable biocompatibility provide ideal nanoprobes for electrochemical immunosensing. For example, Ju's group [57] presented a modular labeling strategy for electrochemical immunoassay via supramolecular host–guest interaction between β-cyclodextrin (β-CD) functionalized AuPd bimetallic nanoparticles (AuPd–CD) and adamantine (ADA) (Fig. 14). The number of ADA moieties loaded on single antibody was calculated to be ~7. After the ADA-Ab was bound to antigen-modified electrode surface with a competitive immunoreaction, AuPd-CD as signal tag was immobilized onto the immunosensor by the host–guest interaction, leading to large loading of AuPd nanoparticles. The highly efficient electrocatalysis of AuPd nanoparticles towards $NaBH_4$ oxidation produced an ultrasensitive response to chloramphenicol as a model of small molecule antigen. The host–guest interaction strategy provided a

Fig. 13 Schematic illustration of hemin@Ng as signal tag for QD-based ECL immunoassay



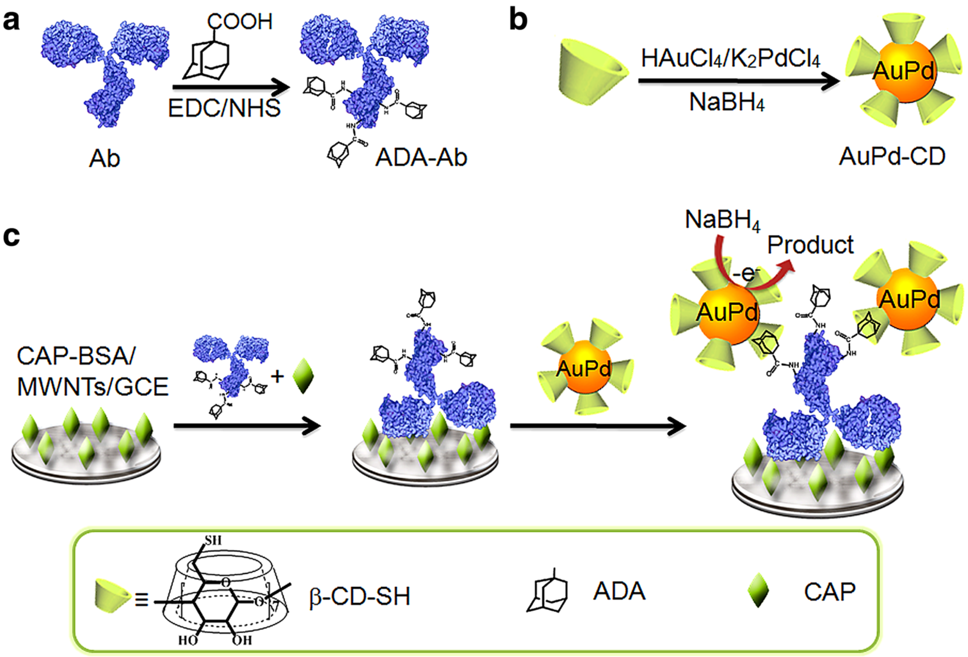


Fig. 14 Schematic representation of the preparation of **a** ADA-Ab conjugate and **b** β-CD functionalized AuPd bimetallic nanoparticles, and **c** electrochemical immunoassay procedure of detection of a small molecule

universal labeling approach for ultrasensitive detection of small molecule targets.

5 Molecular Biological Technology for Signal-Amplified Immunosensing

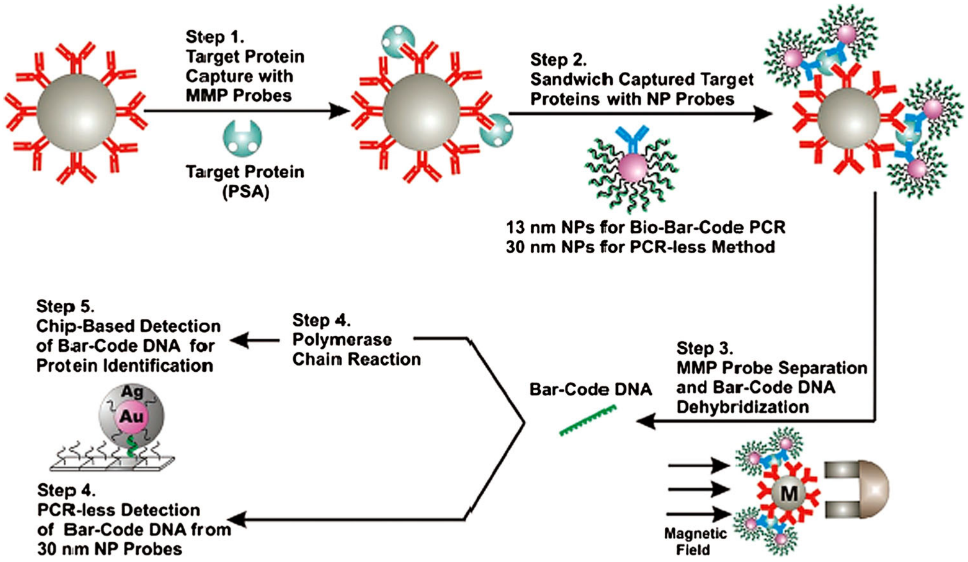
Molecular biological technologies such as polymerase chain reaction (PCR), rolling circle amplification and hybridization chain reaction have extensively been used for signal amplification to develop highly sensitive biodetection methods. The PCR is the earliest applied technique for immunoassay. The developed immuno-PCR methods have been used for detection of biomarkers in clinic medicine. Moreover, these molecular biological technologies can conveniently be combined with the advantages of nanomaterials for development of two dimensional signal amplification strategies, leading to the occurrence of extremely high sensitivity of immunoassay, which can detect several molecules of protein marker with general electrochemical detection [58].

5.1 PCR for Signal-Amplified Immunosensing

In 1992, PCR was firstly introduced into immunoassays by Sano et al. [59]. This technology was named as immuno-PCR or IPCR, which combines the versatility and flexibility of ELISA with the exponential signal amplification power of PCR. Afterward, many researchers have worked to improve the assay workflow of immuno-PCR by focusing on the key aspects of DNA-antibody conjugates, target binding and assay readout. For example, a streptavidin-protein A fusion protein was constructed as a bridge of biotin-DNA complex and antibody based on the specific bind of protein A to the Fc fragment of IgG and streptavidin to the biotin-DNA complex [60]. To overcome this limitation of availability of the fusion proteins and extend the application of immuno-PCR, streptavidin or avidin was used to join both the biotinylated DNA reporter and the biotinylated antibody [61, 62]. Another strategy for constructing antibody-DNA conjugates was direct covalent linkage of the DNA to the antibody, which offered a number of advantages in immuno-PCR [63].

To achieve higher sensitivity, some modified methods of the immuno-PCR have been developed to present multiple reporter DNAs at each binding site. For example, Mirkin's group [64] used short DNA oligonucleotides biofunctionalized nanoparticles as "barcodes" to couple with PCR assay for the identification of protein targets in a homogeneous sandwich (Fig. 15). Gold nanoparticles were immobilized with a polyclonal antibody and a large number of single-stranded DNA molecules. A complementary DNA strand was hybridized to serve as a barcode for the subsequent detection. After a sandwich and magnetic separation, the barcode DNA probes from the nanoparticles that were bound to the proteins were released by dehybridization, and were subsequently identified and quantified by the incorporation of PCR. The assay was able to measure 3 aM of PSA, showing a sensitivity of six orders of magnitude greater than conventional ELISA for PSA. This biobarcode strategy was further used to enhance the

Fig. 15 Overview of the biobarcode immunoassay



sensitivity of IPCR for the detection of Hantaan virus nucleocapsid protein [65].

5.2 Rolling Circle Amplification for Amplified Immunoassay

Rolling circle amplification (RCA) is a simple and efficient isothermal enzymatic process, which synthesizes a long, repetitive ssDNA via special polymerase. A typical RCA reaction requires two reaction steps of ligation and polymerization and can be conducted in solution, on a solid support or in a complex biological environment. T4 DNA ligase helps circularization of DNA template, and Phi29 DNA polymerase initiates an RCA polymerization from the prime-template hybrids, which generate long ssDNA with many tandem copies of the complement to the circularized molecule in a few minutes [66]. Since Ward et al. firstly described an adaptation of the RCA for the detection of protein, termed “immuno-RCA” in 2000 [67], RCA has been explored as important strategies for signal enhancement in immunoassay due to the sensitivity, simplicity, and versatility of the RCA technique [68]. For example, Cheng et al. [58] introduced the biotin–streptavidin system to bind primers to antibody and combined the RCA technique with oligonucleotide functionalized quantum dots (QDs), and anodic stripping voltammetric test, realizing detection of protein target at ultralow concentration (Fig. 16). They used human vascular endothelial growth factor (VEGF) as a model protein to attain an outstanding detection limit of 0.27 aM with a wide linear range from 1 aM to 1 pM. The low detection limit meant that this method could quantitatively detect protein down to 16 molecules in a 100-μL sample. In this work, the RCA and the multiplex binding system presented numerous superior features, including remarkable amplification efficiency, very little nonspecific adsorption, and low background signal.

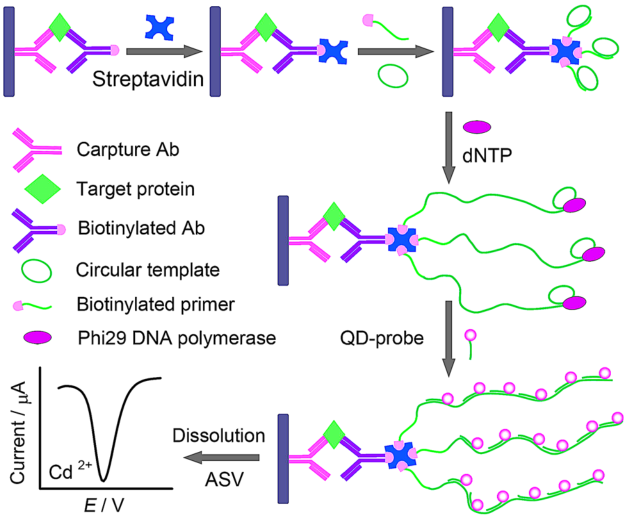


Fig. 16 Schematic representation of the cascade signal amplification strategy for protein detection

Immuno-RCA converted antibody–antigen recognition events to nucleic acid signal amplification. A long DNA production containing hundreds of copies complementary to the circular DNA sequence can be detected via variety of signal-reporting labels. For example, using a highly fluorescent SYBR Green I to intercalate the RCA product, Xue et al. [69] proposed a cascade fluorescence signal amplification strategy based on RCA-aided assembly of fluorescent DNA nanotags as fluorescent labels for detection of protein target at ultralow concentration (Fig. 17). Huang et al. [69] adopted a protein detection technique using surface plasmon resonance (SPR) and RCA. When RCA amplification products characterized by oligonucleotide

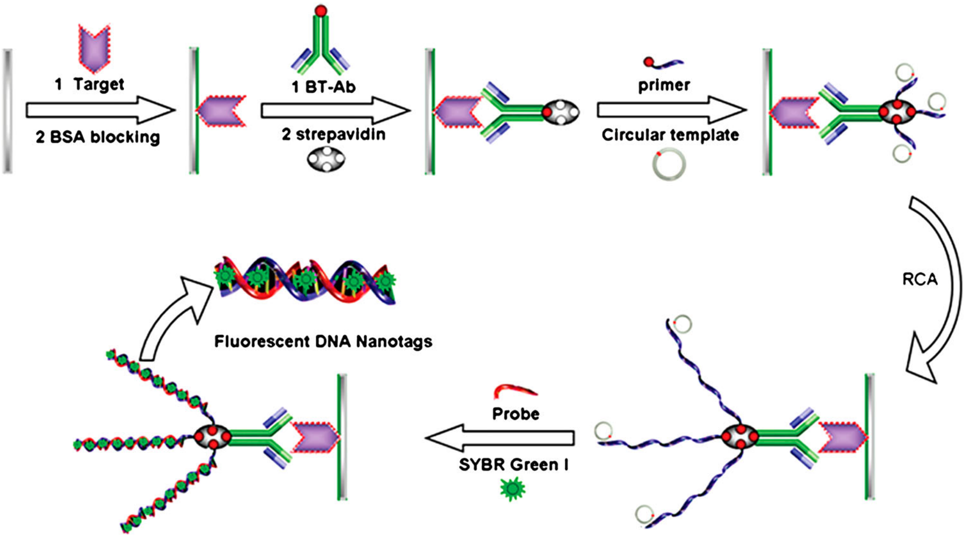


Fig. 17 Illustration of the immunoassay using assembled cascade fluorescent DNA nanotags based on RCA

tags bound to the probes modified on the sensor chip surface, the change of mass on the surface produced the SPR signal for target protein detection.

When the immuno-RCA is coupled with nanoparticle-based amplification format, the sensitivity of immunosensing can be greatly enhanced. For example, AuNPs-modified primers have proven to effectively initiate the RCA reactions. After different epitopes of the target protein were bound to the detection antibody and capture antibody by a specific sandwich immunoreaction, RCA amplification was triggered by AuNPs, which were attached both the detection of antibody and DNA primer [70]. Using biotinylated 2′-deoxyuridine 5′-triphosphate (biotin-dUTP) and avidin–horseradish peroxidase (Av-HRP), RCA products generated enzymatic catalysis-based colorimetric signals, which quantitatively reflected the immunological target recognition event (Fig. 18). This on-nanoparticle rolling-circle amplification (nanoRCA) strategy, combined with the advantages of immuno-RCA and nanoparticle-based assays, allows the visual detection of 30 molecules of CEA, with at least 10^{10}-fold selectivity over a range of noncognate proteins.

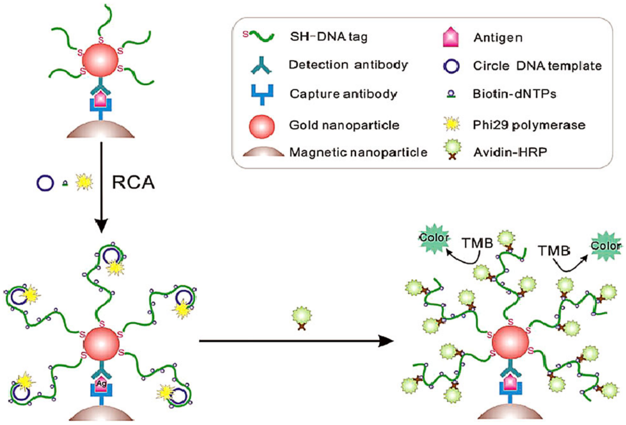


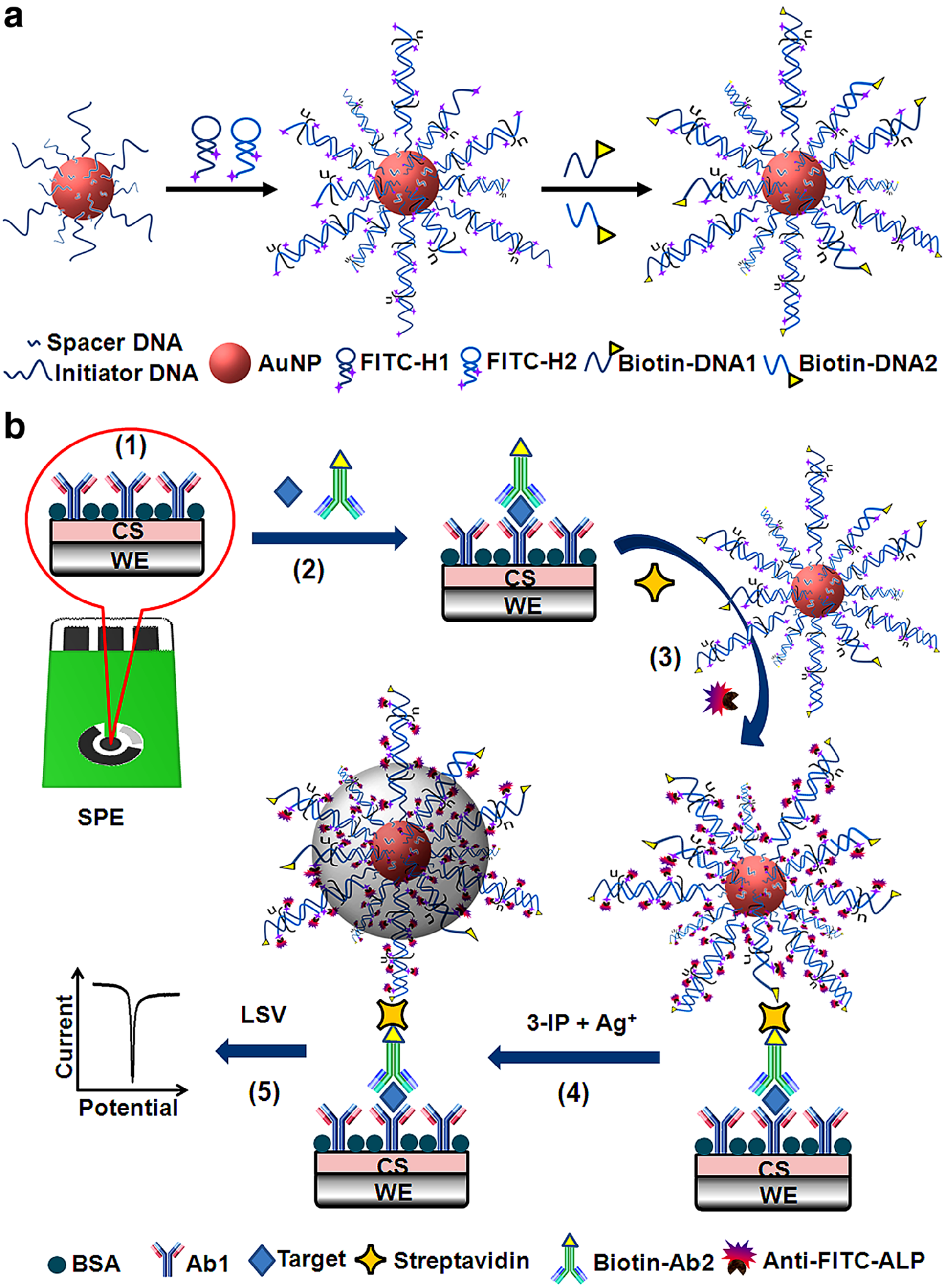
Fig. 18 Schematic illustration of the nanoRCA strategy

5.3 Hybridization Chain Reaction for Amplified Immunoassay

With great attention focused on enzyme-free isothermal amplification strategies, hybridization chain reaction (HCR) amplification shows great potential in signal amplification [71–73]. In HCR, two sets of DNA monomer hairpin structure (H1 and H2) are designed to be partially complementary, which provides a building blocks via amplifying short sequences of oligonucleotides, and achieves an enzyme-free alternative for selective and specific extension at room temperature. The single-stranded DNA that can be programmed to self-assemble into complex structures triggers a chain of alternating hairpin molecules hybridization reaction in which H1 and H2 hairpins sequentially open to assemble into a long nicked double-stranded amplification polymer driven by the free energy of base pair formation [71].

Immuno-HCR offers an attractive route for electrochemical transduction of protein recognition events by combining with electrochemical devices [74]. For example, Ju's group [75] designed a DNA nanopolylinker with high loading of signal molecules as a three-dimensional nanoprobe, which was prepared by rationally engineering dsDNA polymerization on initiator DNA-modified AuNP via an HCR with two kinds of FITC-labeled DNA hairpins (Fig. 19). The biotinylated core–shell nanoprobe was immobilized on the immunosensor surface, and the FITC molecules then bound enzyme labeled anti-FITC antibody to catalyze a silver deposition process via a classic sandwich type and a biotin–streptavidin affinity system. This

Fig. 19 Schematic diagram of the amplified electrochemical immunoassay using the DNA nanopolylinker probe



method showed satisfactory results for linear detection range over 5 orders of magnitude for CEA with a detection limit of 1.2 fg mL^{-1}.

HCR has been coupled with the optical-based immunosensors, including fluorescent [76, 77], electrochemiluminescence [78], and bioluminescence [79] for signal amplification by loading large amounts of reporting molecules. For example, Song et al. [76] introduced streptavidin QDs (SA-QD) as reporting molecules for the detection of PDGF-BB via HCR based on an aptameric system. Wang et al. [80] designed a label-free fluorescence aptasensing strategy using target-triggered HCR amplification and the superior fluorescence quenching ability of GO to the fluorescence of SYBR Green I (SG) as an indicator absorbed to ssDNA (Fig. 20). In the presence of PDGF-BB, helper DNA probe consisting of an aptamer sequence reconfigures to the active Y structure that binds PDGF-BB and subsequently triggers HCR. The SG then bound with the double strands of the HCR product and gave a strong fluorescence signal. This strategy provided a highly sensitive fluorescence detection for PDGF-BB with a limit of detection down to 1.25 pM.

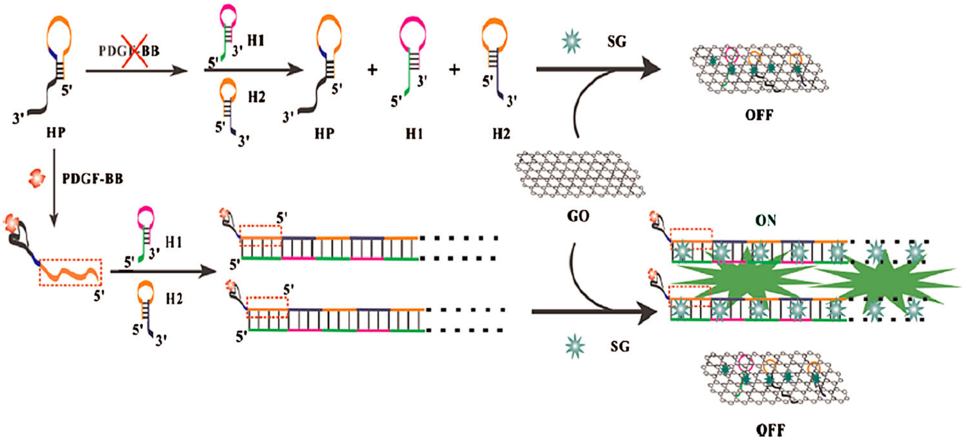


Fig. 20 Schematic illustration of the fluorescence assay for PDGF-BB based on the target-triggered HCR amplification

5.4 Proximity Ligation for Amplified Immunoassay

The proximity ligation assay (PLA) based on proximity hybridization reaction (PHR) is a newly developed DNA-assisted immunoassay technique. This technique makes use of the attractive properties of nucleic acids for desired signal transduction and amplification [81, 82]. For example, Landegren et al. [83] combined proximity ligation assay (PLA) using DNA-bound antibodies to recognize the target molecule with RCA amplification to develop a method for highly sensitive protein detection. Ju et al. [84] designed a ratiometric electrochemical proximity assay for one-step sensitive detection of protein by combination of the ratiometric detection technology with the proximity hybridization. As shown in Fig. 21, upon the proximity hybridization with another antibody-DNA (DNA2-Ab2) triggered by target protein, MB released from the electrode surface and Fc approached to the electrode due to the formation of a hairpin structure, which led to "signal-off" and "signal-on" elements for dual-signal electrochemical ratiometric readout. The electrochemical ratiometry displayed a more sensitive and effective detection than that using single redox label. It extended the linear range for 1 order of magnitude. Further, this group [85] reported an immunoreaction-triggered DNA assembly strategy using proximity hybridization to form a sequence for opening Fc-labeled hairpin DNA (Fc-HDNA) that was immobilized on electrode surface (Fig. 22). The proximity hybridization unfolded the hairpin DNA to form a three-arm DNA structure on the sensing interface, which resulted in the increase and decrease of the electrochemical oxidation signals of Fc and MB, respectively, for ratiometric readout. Using PSA as the target, the logarithmic value of $I_{\mathrm{MB}}/I_{\mathrm{Fc}}$ increased linearly with the increasing logarithm of PSA concentration, leading to a detectable range from 0.01 to 200 ng mL^{-1} with a detection limit of 4.3 pg mL^{-1}.

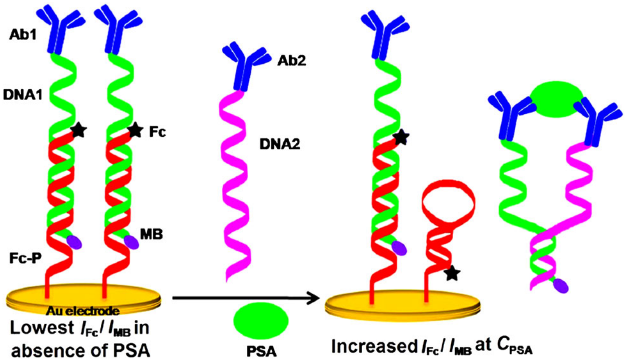


Fig. 21 Schematic illustration of ratiometric electrochemical proximity assay

This technique can be introduced into the formation of DNAzyme as catalytic nucleic acids for signal amplification. The same group [86] integrated the proximity assay and target-driven DNAzyme assembly strategy on a disposable glass chip to develop an array-based chemiluminescence imaging method. The peroxidase-mimicking DNAzyme was split into two fragments (GDNA1 and GDNA2), and each of them was conjugated with Ab or aptamer to prepare a pair of affinity probes. One of the probes was immobilized on the disposable chip. G-quadruplex/hemin DNAzyme was in situ formed after DNA proximity assembly, which generated CL signals for imaging immunoassay of the target proteins.

6 Perspectives

Although tremendous advances have been achieved in the exploring of signal amplification strategies for the development of ultrasensitive immunosensing methods, the detection of specific proteins at ultralow levels in real sample is still particularly challenging. The emergency-bioconjugated nanomaterials have demonstrated the broad potential for the amplified transduction of immunological recognition events. The variability of the preparation of nanomaterials and their

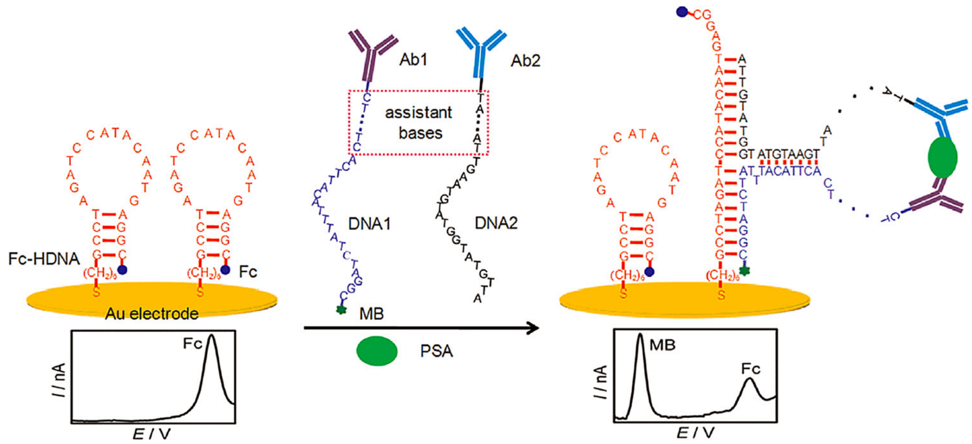


Fig. 22 Schematic illustration of immunoreaction-triggered DNA assembly for ratiometric electrochemical assay of protein biomarker

bio-functionalization often affect the reproducibility and quantification of these immunosensing methods, especially for the real samples. Thus, extensive effort is still an urgent demand to improve the practicability of the nanomaterial-based signal amplification strategies. DNA-based signal amplification techniques have significantly improved the sensitivity of immunoassay using DNA-antibody labeling technologies. Enzyme-free isothermal amplification strategies such as HCR, catalyzed hairpin assembly and DNA-fueled molecular machine may be promising candidates to develop simple and practical signal amplification strategies for protein immunosensing in medicine clinical and diagnosis. The integration of molecular biological amplification and nano amplification strategy will dissolve the bottleneck problem in detection of biomolecule with low abundance and acquisition of ultraweak biological signals. The exploration of new signal amplification techniques with high throughput and high sensitivity is also an urgent demand for the validation and routine application of protein biomarkers.

Acknowledgements We gratefully acknowledge the National Natural Science Foundation of China (21361162002, 21635005), and Priority development areas of The National Research Foundation for the Doctoral Program of Higher Education of China (20130091130005).

References

1. Ju HX. Sensitive biosensing strategy based on functional nanomaterials. Sci China Chem. 2011;54:1202–17.
2. Lei JP, Ju HX. Signal amplification using functional nanomaterials for biosensing. Chem Soc Rev. 2012;41:2122–34.
3. Ju HX, Zhang XJ, Wang J. Nanobiosensing: principles, development and application. New York: Springer Science + Business Media; 2011.
4. Hempen C, Karst U. Labeling strategies for bioassays. Anal Bioanal Chem. 2006;384:572–83.
5. Pei XM, Zhang B, Tang J, Liu BQ, Lai WQ, Tang DP. Sandwich-type immunosensors and immunoassays exploiting nanostructure labels: a review. Anal Chim Acta. 2013;758:1–18.
6. Fenzl C, Hirsch T, Baeumner AJ. Nanomaterials as versatile tools for signal amplification in (bio)analytical applications. TrAC Trends Anal Chem. 2016;79:306–16.
7. Zhou S, Yuan L, Hua X, Xu L, Liu S. Signal amplification strategies for DNA and protein detection based on polymeric nanocomposites and polymerization: a review. Anal Chim Acta. 2015;877:19–32.
8. Lin X, Sun X, Luo S, Liu B, Yang C. Development of DNA-based signal amplification and microfluidic technology for protein assay: a review. TrAC Trends Anal Chem. 2016;80:132–48.
9. Bera D, Qian L, Tseng T, Holloway P. Quantum dots and their multimodal applications: a review. Materials. 2010;3:2260–345.
10. Yuan L, Hua X, Wu YF, Pan XH, Liu SQ. Polymer-functionalized silica nanosphere labels for ultrasensitive detection of tumor necrosis factor-alpha. Anal Chem. 2011;83:6800–9.
11. Yuan L, Xu LL, Liu SQ. Integrated tyramide and polymerization-assisted signal amplification for highly-sensitive immunoassay. Anal Chem. 2012;84:10737–44.
12. Yu X, Munge B, Pate V, Jensen G, Bhirde A, Gong JD, Kim SN, Gillespie J, Gutkind JS, Papadimitrakopoulos F, Rusling JF. Carbon nanotube amplification strategies for highly sensitive immunodetection of cancer biomarkers. J Am Chem Soc. 2006;128:11199–205.
13. Hou L, Cui YL, Xu MD, Gao ZQ, Huang JX, Tang DP. Graphene oxide-labeled sandwich-type impedimetric immunoassay with sensitive enhancement based on enzymatic 4-chloro-1-naphthol oxidation. Biosens Bioelectron. 2013;23:149–56.
14. Lai GS, Cheng H, Xin DH, Zhang HL, Yu AM. Amplified inhibition of the electrochemical signal of ferrocene by enzyme-functionalized graphene oxide nanoprobe for ultrasensitive immunoassay. Anal Chim Acta. 2016;902:189–95.
15. Lai GS, Yan F, Ju HX. Dual signal amplification of glucose oxidase-functionalized nanocomposites as trace label for ultrasensitive simultaneous multiplexed electrochemical detection of tumor markers. Anal Chem. 2009;81:9730–6.
16. Ding L, Bond AM, Zhai J, Zhang J. Utilization of nanoparticle labels for signal amplification in ultrasensitive electrochemical affinity biosensors: a review. Anal Chim Acta. 2013;797:1–12.
17. Huo X, Liu X, Liu J, Sukumaran P, Alwarappan S, Wong DKY. Strategic applications of nanomaterials as sensing platforms and signal amplification markers at electrochemical immunosensors. Electroanalysis. 2016;28:1730–49.
18. Zhang B, Tang D, Liu B, Cui Y, Chen H, Chen G. Nanogold-functionalized magnetic beads with redox activity for sensitive electrochemical immunoassay of thyroid–stimulating hormone. Anal Chim Acta. 2012;711:17–23.

19. Lu W, Cao X, Tao L, Ge J, Dong J, Qian W. A novel label-free amperometric immunosensor for carcinoembryonic antigen based on Ag nanoparticle decorated infinite coordination polymer fibres. Biosens Bioelectron. 2014;57C:219–25.
20. Zhang Q, Prabhu A, San A, Alsharab JF, Levon K. A polyaniline based ultrasensitive potentiometric immunosensor for cardiac troponin complex detection. Biosens Bioelectron. 2015;72:100–6.
21. Johari-Ahar M, Rashidi MR, Barar J, Aghaie M, Mohammadnejad D, Ramazani A. An ultra-sensitive impedimetric immunosensor for detection of the serum oncomarker CA-125 in ovarian cancer patients. Nanoscale. 2015;7:3768–79.
22. Canbaz MÇ, Sezgintürk MK. Fabrication of a highly sensitive disposable immunosensor based on indium tin oxide substrates for cancer biomarker detection. Anal Biochem. 2014;446:9–18.
23. Leng C, Lai GS, Yan F, Ju HX. Gold nanoparticle as an electrochemical label for inherently crosstalk-free multiplexed immunoassay on a disposable chip. Anal Chim Acta. 2010;666:97–101.
24. Cheng H, Lai GS, Fu L, Zhang HL, Yu AM. Enzymatically catalytic deposition of gold nanoparticles by glucose oxidase-functionalized gold nanoprobe for ultrasensitive electrochemical immunoassay. Biosens Bioelectron. 2015;71:353–8.
25. Lai GS, Wu J, Ju HX, Yan F. Streptavidin-functionalized silver-nanoparticle-enriched carbon nanotube tag for ultrasensitive multiplexed detection of tumor markers. Adv Funct Mater. 2011;21:2938–43.
26. Song W, Li H, Liu HP, Wu ZS, Qiang WB, Xu DK. Fabrication of streptavidin functionalized silver nanoparticle decorated graphene and its application in disposable electrochemical sensor for immunoglobulin E. Electrochem Commun. 2013;31:16–9.
27. Jiang XC, Chen K, Wang J, Shao K, Fu T, Shao F, Lu DL, Liang JG, Foda MF, Han HY. Solid-state voltammetry-based electrochemical immunosensor for Escherichia coli using graphene oxide–Ag nanoparticle composites as labels. Analyst. 2013;138:3388–93.
28. Li LH, Feng DX, Zhang YZ. Simultaneous detection of two tumor markers using silver and gold nanoparticles decorated carbon nanospheres as labels. Anal Biochem. 2016;505:59–65.
29. Deng SY, Lei JP, Huang Y, Yao XN, Ding L, Ju HX. Electrocatalytic reduction of coreactant by highly loaded dendrimer-encapsulated palladium nanoparticles for sensitive electrochemiluminescent immunoassay. Chem Commun. 2012;48:9159–61.
30. Lai GS, Yan F, Wu J, Leng C, Ju HX. Ultrasensitive multiplexed immunoassay with electrochemical stripping analysis of silver nanoparticles catalytically deposited by gold nanoparticles and enzymatic reaction. Anal Chem. 2011;83:2726–32.
31. Lin DJ, Wu J, Ju HX, Yan F. Nanogold/mesoporous carbon foam-mediated silver enhancement for graphene-enhanced electrochemical immunosensing of carcinoembryonic antigen. Biosens Bioelectron. 2014;52:153–8.
32. Lin DJ, Wu J, Ju HX, Yan F. Signal amplification for electrochemical immunosensing by in situ assembly of host-guest linked gold nanorod superstructure on immunocomplex. Biosens Bioelectron. 2013;45:195–200.
33. Lin JH, Ju HX. Electrochemical and chemiluminescent immunosensors for tumor markers. Biosens Bioelectron. 2005;20:1461–70.
34. Song WY, Ding L, Chen YL, Ju HX. Plasmonic coupling of dual gold nanoprobes for sers imaging of sialic acids on living cells. Chem Commun. 2016;52:10640–3.
35. Shourian M, Ghourchian H, Boutorabi M. Ultra-sensitive immunosensor for detection of hepatitis B surface antigen using multi-functionalized gold nanoparticles. Anal Chim Acta. 2015;895:1–8.
36. Sabouri S, Ghourchian H, Shourian M, Boutorabi M. A gold nanoparticle-based immunosensor for the chemiluminescence detection of the hepatitis b surface antigen. Anal Methods. 2014;6:5059–66.
37. Li L, Chen Y, Lu Q, Ji J, Shen Y, Xu M, Fei R, Yang G, Zhang K, Zhang J, Zhu J. Electrochemiluminescence energy transfer-promoted ultrasensitive immunoassay using near-infrared-emitting CdSeTe/CdS/ZnS quantum dots and gold nanorods. Sci Rep. 2013;3:776.
38. Wang QG, Yang ZM, Zhang XQ, Xiao X, Chang C, Xu B. A supramolecular-hydrogel-encapsulated hemin as an artificial enzyme to mimic peroxidase. Angew Chem Int Ed. 2007;46:4285–9.
39. Hazarika P, Ceyhan B, Niemeyer CM. Sensitive detection of proteins using difunctional DNA–gold nanoparticles. Small. 2005;1:844–8.
40. Dequaire M, Degrand C, Limoges B. An electrochemical metalloimmunoassay based on a colloidal gold label. Anal Chem. 2000;72:5521–8.
41. Authier L, Grossiord C, Brossier P. Gold nanoparticle–based quantitative electrochemical detection of amplified human cytomegalovirus DNA using disposable microband electrodes. Anal Chem. 2001;73:4450–6.
42. Yang J, Zheng L, Wang Y, Li W, Zhang J, Gu J, Fu Y. Guanine rich DNA-based peroxidase mimetics for colorimetric assays of alkaline phosphatase. Biosens Bioelectron. 2016;77:549–56.
43. Kosman J, Juskowiak B. Peroxidase-mimicking DNAzymes for biosensing applications: a review. Anal Chim Acta. 2011;707:7–17.
44. Travascio P, Li Y, Sen D. DNA-enhanced peroxidase activity of a DNA aptamer–hemin complex. Chem Boil. 1998;5:505–17.
45. Sharon E, Freeman R, Willner I. CdSe/ZnS quantum dots–G-quadruplex/hemin hybrids as optical DNA sensors and aptasensors. Anal Chem. 2010;82:7073–7.
46. Zong C, Wu J, Xu J, Ju HX, Yan F. Multilayer hemin/G-quadruplex wrapped gold nanoparticles as tag for ultrasensitive multiplex immunoassay by chemiluminescence imaging. Biosens Bioelectron. 2013;43:372–8.
47. Xu J, Wu J, Zong C, Ju HX, Yan F. Manganese porphyrin–dsDNA complex: a mimicking enzyme for highly efficient bioanalysis. Anal Chem. 2013;85:3374–9.
48. Wang G, Shu J, Dong Y, Wu X, Li Z. An ultrasensitive and universal photoelectrochemical immunoassay based on enzyme mimetics enhanced signal amplification. Biosens Bioelectron. 2015;66:283–9.
49. Deng SY, Lei JP, Huang Y, Cheng Y, Ju HX. Electrochemiluminescent quenching of quantum dots for ultrasensitive immunoassay through oxygen reduction catalyzed by nitrogen-doped graphene-supported hemin. Anal Chem. 2013;85:5390–6.
50. Gao L, Zhuang J, Nie L, Zhang J, Zhang Y, Gu N. Intrinsic peroxidase-like activity of ferromagnetic nanoparticles. Nat Nanotech. 2007;2:577–83.
51. Wei H, Wang EK. Fe_3O_4 magnetic nanoparticles as peroxidase mimetics and their applications in H_2O_2 and glucose detection. Anal Chem. 2008;80:2250–4.
52. Ma W, Yin H, Xu L, Xu Z, Kuang H, Wang L. Femtogram ultrasensitive aptasensor for the detection of ochratoxin A. Biosens Bioelectron. 2013;42:545–9.
53. Wang C, Qian J, Wang K, Yang X, Liu Q, Hao N, Wang C, Dong X, Huang X. Colorimetric aptasensing of ochratoxin A using Au@ Fe_3O_4 nanoparticles as signal indicator and magnetic separator. Biosens Bioelectron. 2016;77:1183–91.
54. Wei Q, Li T, Wang GL, Li H, Qian ZY, Yang MH. Fe_3O_4 nanoparticles-loaded PEG–PLA polymeric vesicles as labels for ultrasensitive immunosensors. Biomaterials. 2010;31:7332–9.

55. Tseng CW, Chang HY, Chang JY, Huang CC. Detection of mercury ions based on mercury-induced switching of enzyme-like activity of platinum/gold nanoparticles. Nanoscale. 2012;4:6823–30.
56. Hou L, Gao Z, Xu M, Cao X, Wu X, Chen G, Tang D. DNAzyme-functionalized gold–palladium hybrid nanostructures for triple signal amplification of impedimetric immunosensor. Biosens Bioelectron. 2014;54:365–71.
57. Wang LS, Lei JP, Ma RN, Ju HX. Host–guest interaction of adamantine with a β-cyclodextrin-functionalized AuPd bimetallic nanoprobe for ultrasensitive electrochemical immunoassay of small molecules. Anal Chem. 2013;85:6505–10.
58. Cheng W, Yan F, Ding L, Ju HX, Yin YB. Cascade signal amplification strategy for subattomolar protein detection by rolling circle amplification and quantum dots tagging. Anal Chem. 2010;82:3337–42.
59. Sano T, Smith C, Cantor C. Immuno-PCR: very sensitive antigen detection by means of specific antibody–DNA conjugates. Science. 1992;258:120–2.
60. Janssen KPF, Knez K, Spasic D, Lammertyn J. Nucleic acids for ultra-sensitive protein detection. Sensors. 2013;13:1353–84.
61. Ruzicka V, März W, Russ A, Gross W. Immuno-PCR with a commercially available avidin system. Science. 1993;260:698–9.
62. Zhou H, Fisher R, Papas T. Universal immuno-PCR for ultra-sensitive target protein detection. Nucleic Acids Res. 1993;21:6038–9.
63. Joerger R, Truby T, Hendrickson E, Young R, Ebersole R. Analyte detection with DNA-labeled antibodies and polymerase chain reaction. Clin Chem. 1995;41:1371–7.
64. Nam J, Thaxton CS, Mirkin CA. Nanoparticle-based bio-barcodes for the ultrasensitive detection of proteins. Science. 2003;301:1884–6.
65. Chen LY, Wei HP, Guo YC, Cui ZQ, Zhang ZP, Zhang XE. Gold nanoparticle enhanced immuno-PCR for ultrasensitive detection of Hantaan virus nucleocapsid protein. J Immunol Methods. 2009;346:64–70.
66. Kobori T, Matsumoto A, Takahashi H, Sugiyama S. Rolling circle amplification for signal enhancement in ovalbumin detection. Anal Sci. 2009;25:1381–3.
67. Schweitzer B, Wiltshire S, Lambert J, O'Malley S, Kukanskis K, Zhu Z, Kingsmore SF, Lizardi PM, Ward DC. Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. Proc Natl Acad Sci USA. 2000;97:10113–9.
68. Zhao Y, Chen F, Li Q, Wang L, Fan C. Isothermal amplification of nucleic acids. Chem Rev. 2015;115:12491–545.
69. Xue Q, Wang Z, Wang L, Jiang W. Sensitive detection of proteins using assembled cascade fluorescent DNA nanotags based on rolling circle amplification. Bioconjugate Chem. 2012;23:734–9.
70. Yan J, Song S, Li B, Zhang Q, Huang Q, Zhang H, Fan C. An on-nanoparticle rolling-circle amplification platform for ultrasensitive protein detection in biological fluids. Small. 2010;6:2520–5.
71. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. Proc Natl Acad Sci USA. 2004;101:15275–8.
72. Hecht A, Kumar AA, Kopelman R. Label-acquired magnetorotation as a signal transduction method for protein detection: aptamer-based detection of thrombin. Anal Chem. 2011;83:7123–8.
73. Huang J, Wu Y, Chen Y, Zhu Z, Yang X, Yang C, Wang K, Tan W. Pyrene-excimer probes based on the hybridization chain reaction for the detection of nucleic acids in complex biological fluids. Angew Chem Int Ed. 2011;50:401–4.
74. Song C, Xie G, Wang L, Liu L, Tian G, Xiang H. DNA-based hybridization chain reaction for an ultrasensitive cancer marker EBNA-1 electrochemical immunosensor. Biosens Bioelectron. 2014;58:68–74.
75. Tong L, Wu J, Li J, Ju HX, Yan F. Hybridization chain reaction engineered DNA nanopolylinker for amplified electrochemical sensing of biomarkers. Analyst. 2013;138:4870–6.
76. Song W, Zhu K, Cao Z, Lau C, Lu J. Hybridization chain reaction-based aptameric system for the highly selective and sensitive detection of protein. Analyst. 2012;137:1396–401.
77. Dai S, Xue Q, Zhu J, Ding Y, Jiang W, Wang L. An ultrasensitive fluorescence assay for protein detection by hybridization chain reaction-based DNA nanotags. Biosens Bioelectron. 2014;51:421–5.
78. Xiao L, Chai Y, Yuan R, Cao Y, Wang H, Bai L. Amplified electrochemiluminescence of luminol based on hybridization chain reaction and in situ generate co-reactant for highly sensitive immunoassay. Talanta. 2013;115:577–82.
79. Xu Q, Zhu G, Zhang CY. Homogeneous bioluminescence detection of biomolecules using target-triggered hybridization chain reaction-mediated ligation without luciferase label. Anal Chem. 2013;85:6915–21.
80. Wang X, Jiang A, Hou T, Li H, Li F. Enzyme-free and label-free fluorescence aptasensing strategy for highly sensitive detection of protein based on target–triggered hybridization chain reaction amplification. Biosens Bioelectron. 2015;70:324–9.
81. Li F, Lin YW, Le XC. Binding-induced formation of DNA three-way junctions and its application to protein detection and DNA strand displacement. Anal Chem. 2013;85:10835–41.
82. Zhang HQ, Lai MD, Zuehlke A, Peng HY, Li XF, Le XC. Binding-induced DNA nanomachines triggered by proteins and nucleic acids. Angew Chem Int Ed. 2015;54:14326–30.
83. Söderberg O, Leuchowius KJ, Gullberg M, Jarvius M, Weibrecht I, Larsson LG, Landegren U. Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods. 2008;45:227–32.
84. Ren KW, Wu J, Yan F, Ju HX. Ratiometric electrochemical proximity assay for sensitive one-step protein detection. Sci Rep. 2014;4:4360–5.
85. Ren KW, Wu J, Yan F, Zhang Y, Ju HX. Immunoreaction-triggered DNA assembly for one-step sensitive ratiometric electrochemical biosensing of protein biomarker. Biosens Bioelectron. 2015;66:345–9.
86. Zong C, Wu J, Liu MM, Yang LL, Yan F, Ju HX. Chemiluminescence imaging for a protein assay via proximity-dependent DNAzyme formation. Anal Chem. 2014;86:9939–44.