

# Signal Amplification Using Nanomaterials for Biosensing

Jianping Lei and Huangxian Ju

**Abstract** Signal amplification based on biofunctional nanomaterials has recently attracted considerable attention due to the need for ultrasensitive bioassays. Especially, most nanoscaled materials are biocompatible, which permits them to act in direct contact with the environment as carriers of biological recognition elements for obtaining lower and lower detection limit. In order to achieve the good performance for biosensing, two approaches including noncovalent interaction and covalent route have been introduced for the functionalization of nanomaterials with biomolecules. The biofunctional nanomaterials with the abilities of specific recognition and signal triggering can be employed as not only excellent carriers, but also electronic and optical signal tags to amplify the detection signal. These advantages provide a new avenue to construct a sensitive and specific platform in nanobiosensing.

**Keywords** Biosensing, Functionalization, Nanomaterials, Signal amplification

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## 1 Introduction

The need for ultrasensitive bioassays and the trend towards miniaturized assays make the biofunctionalization of nanomaterials become one of the hottest fields [1, 2]. These biofunctionalized nanomaterials can be used as carriers or tracers to obtain the amplified detection signal and the stabilized recognition probes. Based on the unique properties of nanomaterials, the biofunctional nanoparticles can produce a synergic effect among catalytic activity, conductivity, and biocompatibility to result in significantly signal amplification for designing a new generation of nanobiosensing device.

A lot of nanomaterials, such as metal nanoparticles, carbon-based nanostructures, and magnetic nanoparticles have been introduced as carriers for the signal amplification. In particularly, carbon-based nanomaterials and metal nanoparticles show to promote the direct electron transfer between the biomolecules and electrode surface. For example, based on excellent conductivity, the single-walled carbon nanotubes (SWNTs) can act as a nanoconnector that electrically contacts the active site of the enzyme and the electrode with the interfacial electron transfer rate constant of  $42 \text{ s}^{-1}$ , which provides a significant potential for constructing an electrochemical biosensor [3]. Using superparamagnetic particle as carrier for signal amplification, surface plasmon resonance (SPR) immunoassay has been achieved for the detection of cancer biomarker prostate specific antigen (PSA) in serum at an ultralow detection limit of  $10 \text{ fg mL}^{-1}$  [4].

As a signal trace, the biofunctionalized nanomaterials have the abilities of specific recognition and signal amplification in optical, electrochemical, and photoelectrochemical assays [5, 6]. In optical assay, nanoparticle probes such as fluorescence energy transfer nanobeads and quantum dots (QDs) provide significant advantages of signal brightness, photostability, wide dynamic range, and multiplexing capabilities comparison with organic dyes and fluorescent proteins. Electrochemical assays based on nanoprobe are attractive because of their low cost, high sensitivity, simplicity, and easy miniaturization. The electrochemiluminescent (ECL) and photoelectrochemical assays hold the advantages of both optical and electrochemical detections are a promising perspective.

In this chapter, the recent significant advances in signal amplification based on biofunctional nanomaterials are highlighted including the efficient functionalization of nanomaterials with biomolecules as recognition elements, and the functions of nanomaterials as carrier and signal trace in ultrasensitive nanobiosensing.

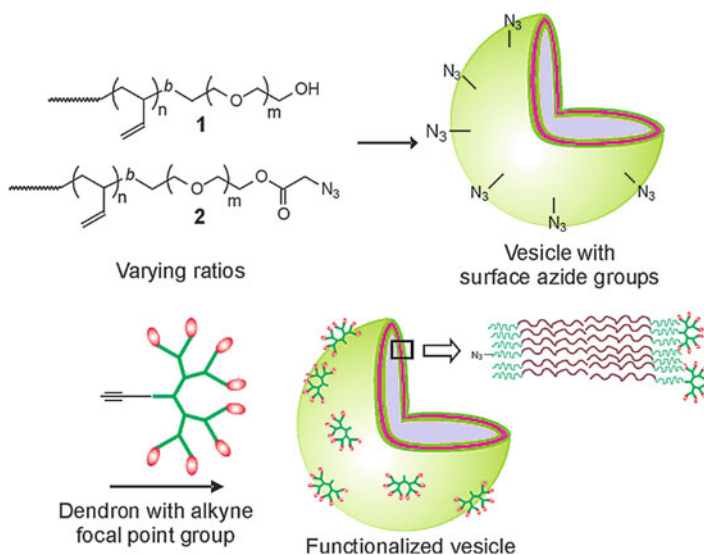
## 2 Biofunctionalization Method of Nanomaterials

### 2.1 Biofunctionalization by Noncovalent Assembly

Nanomaterials hold much promise for biological applications, but they require appropriate functionalization to provide biocompatibility in biological environments. Two approaches including noncovalent interaction and covalent interaction are introduced for the functionalization of nanoparticles (NPs). The noncovalent approach such as electrostatic interaction and  $\pi$ - $\pi$  stacking can avoid destruction of conjugated skeleton and loss of electronic properties of the NPs. A general and attractive approach via  $\pi$ - $\pi$  interaction has been designed by Dai and coworkers for the noncovalent functionalization of SWNTs sidewalls and the subsequent immobilization of biomolecules onto SWNTs via *N*-succinimidyl-1-pyrenebutanoate [7]. A functional nanocomposite of reduced graphene oxide (RGO) with water-soluble picket-fence iron porphyrin has been prepared by means of  $\pi$ - $\pi$  interactions. The resulting nanocomposite has good biocompatibility and excellent electrocatalytic activity toward the reduction of chlorite [8].

The electrostatic interaction is alternative method to assembly biomolecules on the surface of NPs, particularly for deposition of macromolecules such as proteins or enzymes. Typically, the carboxylate group decorated carbon nanotube (CNT) can be functionalized with antibody molecules at pH values that lie slightly above the isoelectric point of the citrate ligand. Further, the electrostatic layer-by-layer (LBL) self-assembly onto CNT carriers maximizes the ratio of enzyme tags per binding event to offer the great amplification factor, which allows detection of DNA and proteins down to 80 copies (5.4 aM) and 2,000 protein molecules (67 aM), respectively [9].

Another noncovalent method for immobilizing biomolecules on NPs is to entrap them in biocompatible films such as phospholipid, polymer, and DNA. A conjugate of phospholipid and dextran has been found to not only be as a stable coating for nanomaterials, but also provide brighter photoluminescence than carbon nanotubes suspended by poly(ethylene glycol) [10]. Moreover, the coating films can provide the abundant positions for functionalization with second biomolecules. Figure 1 shows the formation of polymer vesicles by mixing hydroxyl (1) or azide termini (2) with controlled densities of surface azide groups. Dendrons with focal point alkynes can subsequently be conjugated to the surface azides providing controlled densities of dendritic groups on the vesicle surface. The dendritic systems exhibit one to two orders of magnitude enhancement in binding affinity relative to the



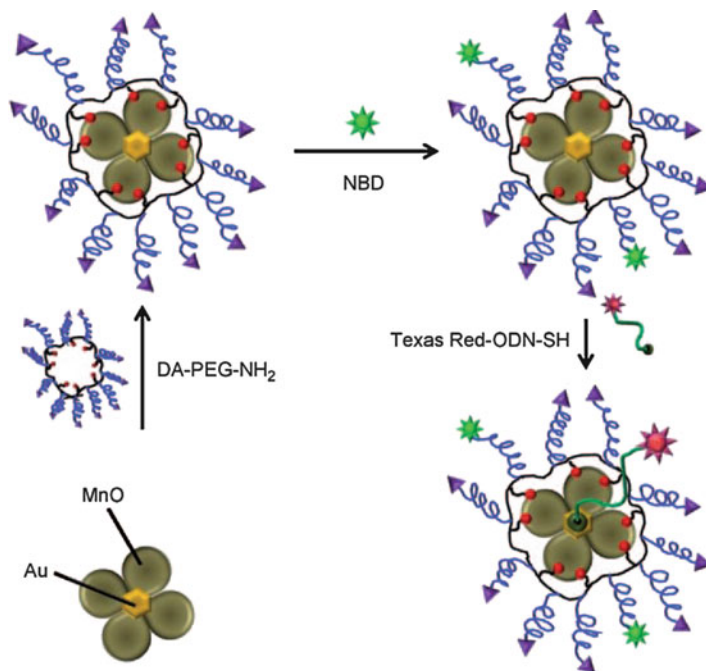
**Fig. 1** General approach for functionalization of vesicle surfaces with dendritic groups. Reprinted with permission from Martin et al. [11]. © 2009, American Chemical Society

nondendritic displays, which is attributed to the ability of the dendritic groups to overcome steric inhibition by polymer chains at the material surface and also to the presentation of ligands in localized clusters [11]. A new strategy for the synthesis of metal–nanoparticle/CNT nanohybrids has been developed by ionic-liquid polymer (PIL)-functionalized CNTs. The PtRu/CNTs-PIL electrocatalyst shows better performance in the direct electrooxidation of methanol than the PtRu/CNTs electrocatalyst alone [12].

Affinity interactions, such as antigen–antibody, nucleic acid–DNA, lectin–glycan, streptavidin–biotin, and aptamer–protein, are highly stable and the strongest of all noncovalent linkages for bioconjugation of targeting ligands to NPs. Moreover, various biomolecules contain several binding sites, for example, streptavidin or concanavalin A, each displays four binding domains. This allows the multidirectional growth of NPs structures [13, 14]. In addition, barnase–barstar system is a new generic method for robust self-assembly of multifunctional particles to macroscopic superstructures [15].

## 2.2 Covalent Route

Biofunctionalization of nanomaterials employing covalent methods should be preferable to unspecific physisorption in terms of stability and reproducibility of the surface functionalization. In general, functional groups at the NP surfaces can be directly bound to reactive ligands by a linkage reaction facilitated with the aid of



**Fig. 2** Surface functionalization of Au@MnO nanoflowers with a multidentate copolymer and subsequent conjugation with NBD. The gold domain was selectively functionalized with a Texas-Red-tagged thiolated oligonucleotide. Reprinted with permission from Schladt et al. [20]. © 2010, Wiley

catalysts. Typically, CNTs can be firstly shortened by sonication in 3:1 H<sub>2</sub>SO<sub>4</sub>/HNO<sub>3</sub> for several hours refluxing to introduce hydrophilic carboxylic acid groups for functionalization. Then NPs decorated with carboxylic acid groups can be covalently bound to biomolecules bearing primary amines through *N*-hydroxysuccinimide linkers [16]. On the basis of the arginine–glycine–aspartic acid–serine (RGDS)-functionalized SWNTs, a novel electrochemical cytosensing strategy has been designed with detection limit down to 620 cells mL<sup>−1</sup> and linear calibration range from  $1.0 \times 10^3$  to  $1.0 \times 10^7$  cells mL<sup>−1</sup> of BGC-823 human gastric carcinoma cells [17]. Similarly, arginine–glycine–aspartic acid-labeled QDs have also been designed for in vivo targeting and imaging of tumor vasculature [18]. The tumor fluorescence intensity reaches maximum at 6 h postinjection with good contrast.

As to metal nanoparticles, the primary binding of thiolated molecules, such as thiolated oligopeptides, to gold nanoparticles (AuNPs) can provide a means for the covalent tethering of biomolecules to NPs [19]. Figure 2 depicts a functionalized Au@MnO nanoflower with selective attachment of catechol anchors to the metal oxide petals and thiol anchors to the gold core. Selective functionalization of the gold domain can be achieved by incubating an aqueous solution of the fluorescent

dye 4-chloro-7-nitrobenzofurazan (NBD)-polymer-modified Au@MnO NPs with thiol-modified 24-mers customized oligonucleotide tagged with Texas red. The polymer-functionalized Au@MnO NPs are stable against aggregation and precipitation in various aqueous media, including deionized water and PBS buffer solution for several days. Viability assays of nanocomposite solutions with the renal cell carcinoma line Caki<sup>-1</sup> show negligible toxicity of the nanoparticles even for concentrations as high as 140 mg mL<sup>-1</sup> [20]. A DNA sensor based on a “sandwich” detection strategy has been designed, which involves capture probe DNA immobilized on gold electrodes and reporter probe DNA labeled with AuNPs via Au–S chemistry [21].

Some specific reactions under mild reaction conditions have been extensively used in the generation of covalent-tethered conjugates of biomolecules with various NPs. To design a modular and broadly applicable targeting platform, Weissleder and coworkers described a covalent bioorthogonal reaction between a 1,2,4,5-tetrazine and a trans-cyclooctene for small-molecule labeling. The [4+2] cycloaddition was fast, chemoselective, did not require a catalyst, which was adapted to targeting nanoparticle sensors in different configurations to improve binding efficiency and detection sensitivity [22]. On the other hand, “Click” chemistry, a Cu-catalyzed azide–alkyne cycloaddition, is a relatively new approach for easy and almost quantitative functionalization with high specificity, high stability, and extreme rigidity [23]. Via one-step Click reaction, the drug-loaded polymer nanoparticles can be functionalized with folate, biotin, and gold nanoparticles for drug delivery [24]. A general approach has been presented for functionalization of low-fouling, nanoengineered polymer capsules with antibodies by using click chemistry. Significantly, antibody-functionalized capsules can specifically bind to colorectal cancer cells even when the target cells constitute less than 0.1% of the total cell population [25].

### 3 Nanomaterials as Carriers for Signal Amplification

#### 3.1 *Metal Nanoparticles*

Metal NPs have been extensively used in the detection of biologically important or toxic substances, usually by the change of spectral or SPR signal accompanying NP aggregation or dispersion in the presence of an analyte. Using AuNPs as carriers, a homogeneous colorimetric DNA biosensor has been developed by a novel nicking endonuclease-assisted AuNP amplification, resulting in a 103-fold improvement in amplification (ca. 10 pm) and the capability of recognizing long single-stranded oligonucleotides with single-base mismatch selectivity [26]. NP supracrystals and core–shell supracrystals stabilized by analyte-specific cross-linkers can enhance dramatically (by over two orders of magnitude compared to noncrystalline NP aggregates) the sensitivity of NPs-based detection [27].

A dual element amplification method based on AuNPs and RNA transcription is designed by using SPR imaging to detect single-stranded DNA (ssDNA) down to a concentration of 1 fM in a volume of 25  $\mu\text{L}$  (25 zeptomoles) [28]. Surface plasmons (SP)-induced ECL enhancement has been applied for ultrasensitive detection of thrombin with the concentration of thrombin in a wide range from 100 aM to 100 fM. This system shows five fold enhancement of ECL intensity as compared to that without AuNPs, which might be attributed to the long-distance interaction between the semiconductor nanocrystal and SPR field of noble metal NPs [29].

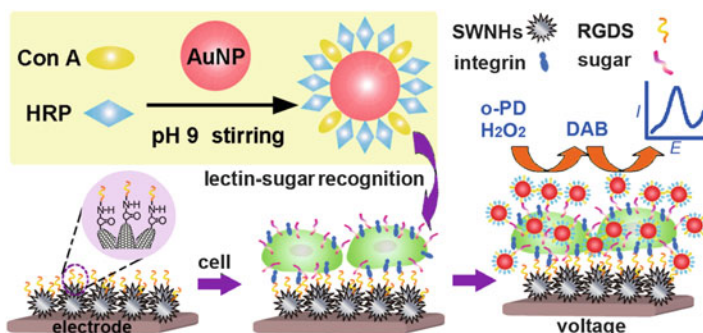
Due to the unique electrochemical properties, AuNPs can significantly enhance the sensitivity and the selectivity in the electrochemical detection of DNA. DNA-functionalized AuNPs (DNA-AuNPs) have been used to enhance the sensitivity of the aptasensor because DNA-AuNPs-modified interface can load more  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  cations to produce electrochemical signal. The proposed aptasensor has a low detection limit (0.02 nM for adenosine and 0.01  $\mu\text{g mL}^{-1}$  for lysozyme) [30]. A highly selective electrochemical biosensor for the ultrasensitive detection of  $\text{Hg}^{2+}$  in aqueous solution has been developed based on the strong and specific binding of  $\text{Hg}^{2+}$  by two DNA thymine bases (T- $\text{Hg}^{2+}$ -T) and the use of AuNP-functionalized reporter DNA to achieve signal amplification [31, 32].

### 3.2 Carbon-Based Nanomaterials

Carbon-based NPs are excellent carriers to enhance the probe due to the good conductivity and biocompatibility. Typically, SWNTs have been used to immobilize DNA probe for fabrication of DNA biosensor. Based on the direct current response of guanine, the biosensor can detect target DNA in the range of 40–110 nM with a detection limit of 20 nM [33]. Ready renewal for more than 3,000 times is the outstanding merit of this label-free biosensor. Moreover, CNTs play a dual amplification role in both the recognition and transduction events, namely as carriers for numerous enzyme tags and for accumulating the product of the enzymatic reaction. Using alkaline phosphatase (ALP)-CNTs as a tracer, a favorable response of DNA target indicates a remarkably low detection limit of around 1 fg  $\text{mL}^{-1}$  (54 aM), i.e., 820 copies or 1.3 zmol in the 25  $\mu\text{L}$  sample [34]. Based on the specific recognitions of target DNA and streptavidin to biotin-labeled molecular beacon and the signal amplification of streptavidin–horseradish peroxidase (HRP)-functionalized CNTs, a biosensing strategy has been developed for selective electrochemical detection of DNA in five orders of magnitude with a detection limit of 2.8 aM [35].

Compared with SWNTs, single-walled carbon nanohorns (SWNHs) as immobilization matrixes show a better sensitizing effect. This material has been used as carrier to develop an immunosensor for microcystin-LR (MC-LR) ranging from 0.05 to 20  $\mu\text{g L}^{-1}$  [36]. A nanoscaffold of nanohorns functionalized with RGDS has also been prepared on an electrode surface for cell capture and enhancing the electrical connectivity (Fig. 3). Combined with the AuNPs-Con A-HRP nanoprobe





**Fig. 3** Scheme of nanoprobe assembly and electrochemical strategy for in situ detection of mannose groups on living cells. Reprinted with permission from Ding et al. [37]. © 2010, American Chemical Society

and peptide-functionalized nanohorns, a highly sensitive electrochemical strategy is developed for cytosensing, which shows a detection limit down to 15 cells, broad dynamic range, acceptable rapidity, and low cost [37].

Nitrogen-doped carbon nanotubes (CNx-MWNTs) are suitable for loading biomolecules to construct biosensors due to lower cytotoxicity and better biocompatibility. An AuNPs/CNx-MWNTs nanocomposite has been used as an immobilization scaffold of antibodies for preparation of a sensitive immunosensor to detect MC-LR. The immunosensor exhibits a linear response to MC-LR ranging from 0.005 to 1  $\mu\text{g L}^{-1}$  with a detection limit of 0.002  $\mu\text{g L}^{-1}$  at a signal-to-noise of 3 [38].

The functionalized carbon nanospheres (CNSs) are often used for the biosensor platform to increase the surface area for capturing a large amount of primary antibodies, thus amplifying the detection response. For example, the AuNPs/CNSs hybrid material can be conjugated with HRP-labeled antibody (HRP-Ab<sub>2</sub>) to fabricate HRP-Ab<sub>2</sub>-AuNPs/CNSs bioconjugates, which can then be used as a label for the sensitive detection of human IgG (HIgG). This approach provides a linear response range between 0.01 and 250  $\text{ng mL}^{-1}$  with a detection limit of 5.6  $\text{pg mL}^{-1}$  [39]. On the basis of the dual signal amplification strategy of graphene sheets and the multienzyme labeling on CNSs, an immunosensor shows a seven fold increase in detection signal compared to the immunosensor without graphene modification and CNSs labeling. The proposed method can respond to 0.02  $\text{ng mL}^{-1}$   $\alpha$ -fetoprotein (AFP) with a linear calibration range from 0.05 to 6  $\text{ng mL}^{-1}$  [40].

Functionalized graphene oxide (GO) sheets coupled with the nanomaterial-promoted reduction of silver ions have been developed for the sensitive and selective detection of bacteria. Using an electrochemical technique, a linear relationship between the stripping response and the logarithm of the bacterial concentration is obtained for concentrations ranging from  $1.8 \times 10^2$  to  $1.8 \times 10^8$   $\text{cfu mL}^{-1}$  [41]. Based on the supramolecular assembly of free-base cationic 5,10,15,20-tetrakis (1-methyl-4-pyridinio)porphyrin on reduced graphene, the resulting graphene-porphyrin hybrid as an optical probe has been constructed for rapid and selective sensing of  $\text{Cd}^{2+}$  ions in aqueous media [42].



### 3.3 Magnetic Nanoparticles

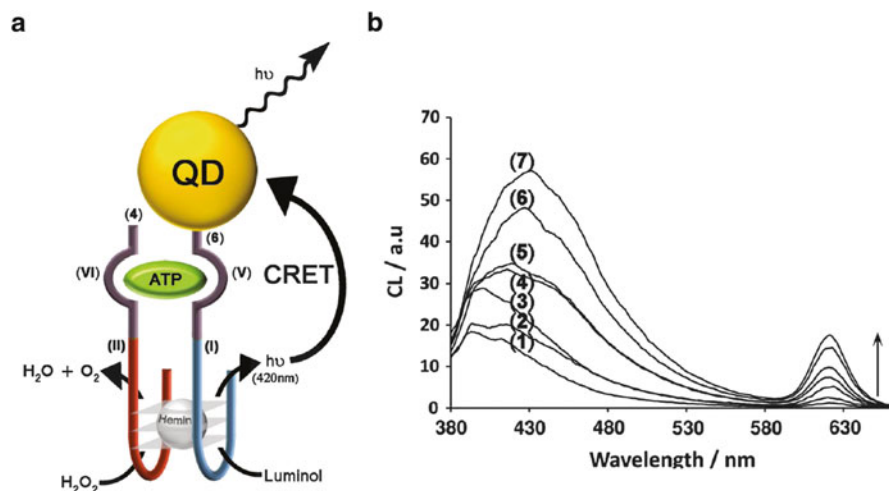
Magnetic NPs easily achieve concentration and purification of analysts, which is useful to enhance dramatically the sensitivity in biosensing. Using p19-functionalized magnetic beads, over 100,000-fold enrichment of the probe:miRNA duplex has been achieved from total RNA. This approach is validated by detecting picogram levels of a liver-specific miRNA (miR122a) from rat liver RNA [43]. The antiferromagnetic NPs have been used to enable magnetic detection of biomolecules at low analyte concentrations (10 pm) with better detection than conventional superparamagnetic materials [44].

Magnetic beads are the good candidates as supporters for largely loading signal trace in ultrasensitive detection. The sensitivity can greatly be amplified by synthesizing magnetic bioconjugates particles containing 7,500 HRP labels along with detection antibodies ( $Ab_2$ ) attached to activated carboxyl groups on 1  $\mu\text{m}$  diameter magnetic beads. The resulting sensor shows a sensitivity of  $31.5 \mu\text{A mL ng}^{-1}$  and a detection limit of  $0.5 \text{ pg mL}^{-1}$  for PSA in 10  $\mu\text{L}$  of undiluted serum [45]. When coupled to superparamagnetic beads massively loaded with about 500,000 HRP labels and  $Ab_2$ , an unprecedented detection limit has been obtained to be  $1 \text{ fg mL}^{-1}$  (100 am) for interleukin 8, which is lower than that of any method for direct biomarker protein detection in serum [46]. The near-single-protein sensor has great promise for extension to arrays for clinical cancer screening and therapy monitoring.

Iron oxide ( $\text{Fe}_3\text{O}_4$ ) nanoparticles, as the well-known hard magnetic material, have been extensively applied in the nanoparticle-based assays due to good biocompatibility. With the employment of the AuNPs–Prussian blue (PB)– $\text{Fe}_3\text{O}_4$  nanohybrid, a signal amplification strategy has been developed based on bienzyme (HRP and glucose oxidase) functionalized Au–PB– $\text{Fe}_3\text{O}_4$  NPs for electrochemical immunosensing. The linear ranges span the concentrations of carcinoembryonic antigen (CEA) from 0.01 to  $80.0 \text{ ng mL}^{-1}$  with detection limit of  $4 \text{ pg mL}^{-1}$  and AFP from 0.014 to  $142.0 \text{ ng mL}^{-1}$  with detection limit of  $7 \text{ pg mL}^{-1}$ , respectively [47]. A sandwich-type electrochemical immunoassay has been designed for the detection of carbohydrate antigen 125 (CA125) using anti-CA125-coated magnetic beads for target capture and HRP-anti-CA125-coated silica beads containing HRP and thionine for signal enhancement. This immunoassay exhibits a range from 0.1 to  $450 \text{ U mL}^{-1}$  with a detection limit of  $0.1 \text{ U mL}^{-1}$  for CA125 [48].

A luminol– $\text{H}_2\text{O}_2$ –HRP–bromophenol blue chemiluminescence (CL) system has been applied to a sandwich-type CL immunoassay based on the magnetic separation and the amplification feature of AuNPs as HRP labels. The linear range for AFP is from 0.1 to  $5.0 \text{ ng mL}^{-1}$  with the detection limit of  $0.01 \text{ ng mL}^{-1}$ , which is one order of magnitude lower than that obtained without using AuNPs [49]. A sensitive strategy, which integrates a DNA cycle device onto magnetic microbeads, amplifying the signal with GO and enhancing ECL intensity, has successfully been applied to thrombin detection [50].

Magnetic nanotags are a promising alternative as giant magnetoresistive sensors, such as spin valve sensors in biomolecular detection assays. With the addition of magnetic nanotag amplification, an inexpensive giant magnetoresistive sensor has



**Fig. 4** (a) Analysis of ATP through the CRET from luminol, oxidized by the assembled hemin/G-quadruplex, to the QDs. (b) Luminescence spectrum corresponding to the CRET signal of the QDs at  $\lambda = 612$  nm in the absence of ATP, curve (1), and in the presence of different concentrations of ATP: (2) 0.125, (3) 1.25, (4) 5, (5) 12.5, (6) 50, (7) 100  $\mu$ M. Reprinted with permission from Freeman et al. [54]. © 2011, American Chemical Society

been constructed for multiplex protein detection of potential cancer markers at subpicomolar concentration levels and with a dynamic range of more than four decades [51]. In addition, combining with two-color photo-acoustic flow cytometry, a platform using targeted magnetic nanoparticles has been developed for in vivo magnetic enrichment and detection of rare circulating tumor cells from a large pool of blood with high spatial resolution [52].

### 3.4 Other Nanomaterials

QDs are most frequently used semiconductor nanoparticles for biological detection of DNA. By integrating CdTe QDs with different biomolecules, such as molecular beacon (MB) and aptamer, an effective sensing platform has been designed for DNA target based on fluorescence resonance energy transfer (FRET) between QDs and graphene oxide. The change in fluorescent intensity is used for the detection of the target with a detection limit down to 12 nM [53].

Most recently, Willner and coworkers implemented the DNAzyme-stimulated chemiluminescence resonance energy transfer (CRET) to CdSe/ZnS QDs for developing aptamer or DNA sensing platforms [54]. Figure 4 depicts the CRET-based analysis of adenosine-5'-triphosphate (ATP) by the hemin/G-quadruplex conjugated aptasensor. Glutathione (GSH)-capped CdSe/ZnS QDs ( $\lambda_{em} = 620$  nm) are covalently tethered to the thiol-functionalized nucleic acid (6) (average loading

ca. 10 units per particle), which consists of the anti-ATP aptamer subunit (V), and the HRP-mimicking DNAzyme subunit (I). Treatment of the (6)-functionalized QDs with ATP in the presence of the nucleic acid (4) that includes the complementary aptamer subunit, region (VI), and the second DNAzyme subunit (II) resulted in the formation of the ATP hemin/G-quadruplex-QDs complex. Figure 4b shows that, upon the addition of ATP, the resulting chemiluminescence stimulates a CRET process, which is intensified by the luminescence of the QDs,  $\lambda_{em} = 620$  nm. Thus ATP can be detected with a sensitivity corresponding to 100 nM.

Due to the small size, high surface-to-volume ratio and good biocompatibility, silica NPs have become another normally used carrier for signal amplification. Labels based on mesoporous silica nanoparticles (MSN) loaded with mediator thionine (TH), HRP, and Ab<sub>2</sub> have been developed in order to improve the sensitivity of an amperometric immunosensor [55]. The sensitivity of the sandwich-type immunosensor using MSN-TH-HRP-Ab<sub>2</sub> as labels for HlgG detection is about 100 times higher than that using either MSN-TH-Ab<sub>2</sub> or MSN-HRP-Ab<sub>2</sub> as labels, indicating the high catalytic efficiency of HRP in the presence of mediator TH toward H<sub>2</sub>O<sub>2</sub>.

Based on dual signal amplification of poly-(guanine)-functionalized silica nanoparticles label and Ru(bpy)<sub>3</sub><sup>2+</sup>-induced catalytic oxidation of guanine, an electrochemical immunosensor for the detection of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is presented. The detection limit for TNF- $\alpha$  is found to be  $5.0 \times 10^{-11}$  g mL<sup>-1</sup> (2.0 pM), which corresponds to 60 amol of TNF- $\alpha$  in 30  $\mu$ L of sample [56]. The ECL of Ru(bpy)<sub>3</sub><sup>2+</sup> doped on silica nanoparticles is more than 1,000-fold increase than that of a single dye, suggesting that the use of this kind of nanostructures as luminescent labels represents a very promising system for ultrasensitive bioanalysis [57].

## 4 Functional Nanomaterial-Amplified Optical Assay

### 4.1 Colorimetric Detection of Biological Analytes

Colorimetric sensors are particularly important because they minimize or eliminate the necessity of using expensive and complicated instruments. Among the many colorimetric sensing strategies, metallic nanoparticle-based detection is desirable because of the high extinction coefficients and strong distance-dependent optical properties of the NPs. For example, colorimetric detection of DNA sequences based on electrostatic interactions with unmodified AuNPs can be completed within 5 min, and <100 fmol of target produces color change observable without instrumentation [58].

The colorimetric bio-barcode assay is a red-to-blue color change-based protein detection method with ultrahigh sensitivity. This assay is based on both the bio-barcode amplification method that allows for detecting miniscule amount of targets with attomolar sensitivity and AuNPs-based colorimetric DNA detection method that allows for a simple and straightforward detection of interleukin-2 [59]. Since AuNPs folded with aptamer are more stable toward salt-induced aggregation than

those unfolded aptamers, colorimetric biosensors have been developed for the detection of adenosine,  $K^+$ , adenosine deaminase, and its inhibitors [60].

Based on detecting  $Cu^{II}$  released from copper monoxide nanoparticle-labeled antibodies as  $Ab_2$  via click chemistry, a colorimetric immunoassay has been developed for the detection of human immunodeficiency virus with the naked eye [61]. This method is highly specific even in the presence of high concentrations of mixtures of other cations and interfering molecules.

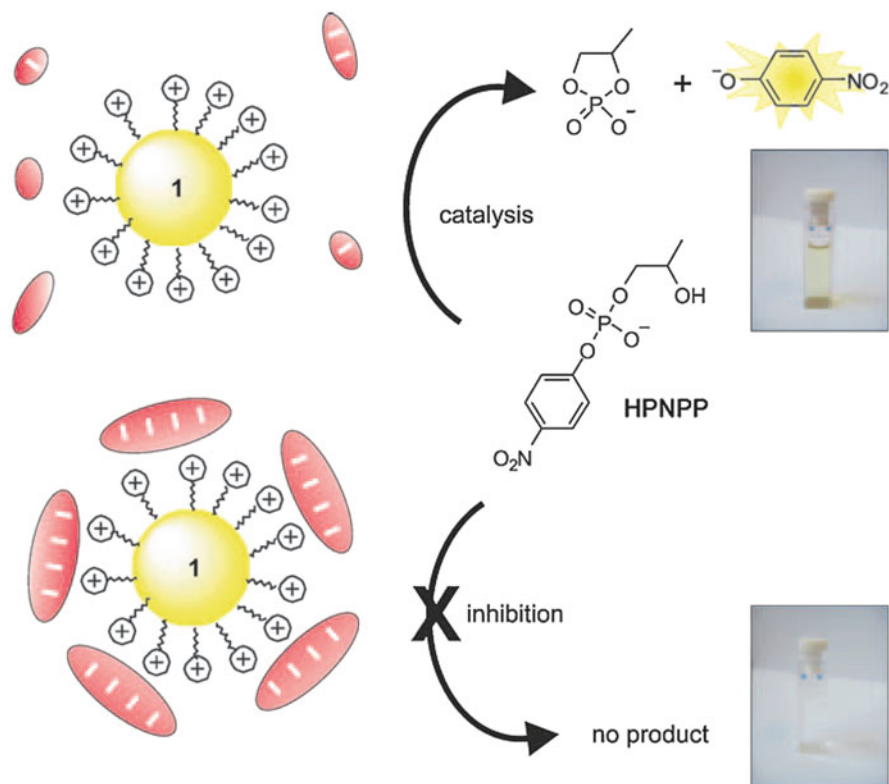
The greater signal enhancement for colorimetric detection can be obtained by catalytic deposition of gold or silver NPs. For example, a convenient and label-free scanometric approach for DNA assay has been designed by integrating a metal ion-mediated conformational MB and silver-signal amplification regulated by AuNPs aggregation. By using scanometric detection, the concentration of the target DNA sequence can be conveniently read out within a linear range from 1.0 to 30 nM [62]. A PCR-free colorimetric assay has been developed for telomerase activity that relies on polyvalent oligonucleotide–nanoparticle conjugates as probes and the concept of elongated and unmodified oligonucleotides on one particle for amplification. The assay can detect telomerase activity with as few as 10 HeLa cells, with on-chip positive and negative controls [63]. An information transfer strategy has been developed for the visualization of carbohydrate expression by the competition of a primary cell-adhered solid surface with a carbohydrate assembled surface as an artificial secondary surface for one species. The strategy can be effectively utilized for in situ monitoring of dynamic carbohydrate expression on an adhesive cell surface [64]. Further, an ultrasensitive glycans array using iron oxide/gold core/shell nanoparticles conjugated with antibodies or proteins has been developed for ultrasensitive detection of carbohydrate–protein interactions [65].

Based on the catalytic activity of AuNPs covered with a self-assembled organic monolayer (Au-MPC) toward 2-hydroxypropyl-4-nitrophenylphosphate (HPNPP), a catalytic amplification process has been developed for the detection of proteases (Fig. 5). The strategy relies on a cascade of two catalytic events for signal generation. In the first event, an enzyme hydrolyzes a peptide substrate, which acts as an inhibitor for the catalytic monolayer. Upon hydrolysis, the catalytic activity of the monolayer is restored, and large quantities of a yellow reporter molecule are produced, leading to a sensitive colorimetric assay for the detection of enzyme activity [66].

In order to achieve an amplification of the optical signal, AuNPs have been used as carriers of the signaling HRP-anti-CA153 for the immunoassay of CA153 antigen. In the range up to  $60\text{ U mL}^{-1}$ , the assay adopting AuNPs as an enhancer results in higher sensitivity and shorter assay time when compared to classical enzyme-linked immunosorbent assay [67].

## 4.2 Fluorescence Detection

Fluorescence detection is currently one of the most widely used methods in the areas of biotechnology, medical testing, and drug discovery. Based on the fluorescence signal recovery after digestion of RNA by RNase H, a strategy of



**Fig. 5** Schematic representation of the catalytic assay. The presence of an enzyme able to hydrolyze a substrate enables catalysis of the transphosphorylation of HPNPP by Au-MPC resulting in the release of a yellow reporter molecule. In the absence of the enzyme, the catalytic activity of Au-MPC is suppressed because the enzyme substrate acts as an inhibitor for Au-MPC. Reprinted with permission from Bonomi et al. [66]. © 2011, Wiley

fluorescence signal amplification has been developed for highly sensitive and rapid protease assay at concentrations as low as 10 pM within 4 h [68]. A fluorescence-quenched peptide-based AuNP probes has been developed to visualize proteolytic activity in vivo. Optimal AuNP probes targeted to trypsin and urokinasetype plasminogen activator require the incorporation of a dark quencher to achieve five- to eight fold signal amplification [69]. In addition, a much simpler and milder strategy to amplify fluorescence signal has been proposed by the cation-exchange reaction with ionic nanocrystals. The  $\text{Cd}^{2+}$  released from CdSe QDs can trigger the fluorescence of dyes and lead to a 60-fold enhancement of the fluorescence signal and a limit of detection in protein detection 100 times lower than that of the organic fluorophore Alexa 488 [70].

Based on dual-color imaging and automated colocalization of bioconjugated nanoparticle probes, routine two-color super resolution imaging and single-molecule detection have been achieved at nanometer precision with standard

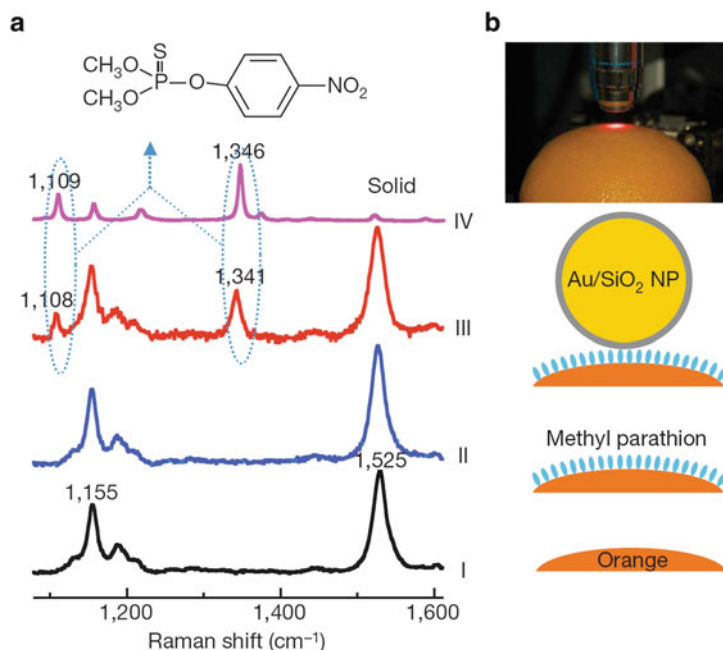
fluorescence microscopes and inexpensive digital color cameras. This approach can apply in single-molecule studies in cell lysate samples with a detection dynamic range over three orders of magnitude [71].

FRET is a good opportunity to set up an ultrasensitive and reliable nanotechnology assay. This approach based on QDs FRET can detect as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles and allows for multiplexed analyses [72]. FRET-based probes incorporated with single-molecule fluorescence detection technologies can allow detection of DNA with low abundance without additional amplification. Unbound nanosensors produce near-zero background fluorescence, but binding to even a small amount of target DNA (~50 copies or less) can generate a very distinct FRET signal. The detection limit is 100-fold greater than conventional FRET probe-based assays as monitored by confocal fluorescence spectroscopy [13]. A bottom-up strategy has been developed to construct water-soluble fluorescent single-molecular nanoparticles based on polyhedral oligomeric silsesquioxanes (POSS) and cationic oligofluorene for fluorescence amplification in cellular imaging. The fluorescence of intercalated ethidium bromide is substantially amplified by 52-fold upon excitation of cationic oligofluorene substituted POSS in buffer, allowing naked-eye discrimination of dsDNA from ssDNA [73]. A plasmonic- and FRET-based DNA sensing scheme has also been designed based on core-shell multilayer dye-doped acceptor nanoparticles grafted with ssDNA probes and complexed with a cationic conjugated polymer, resulting in direct molecular detection of target nucleic acids at femtomolar concentrations [74].

An ECL resonance energy transfer (ECL-RET) system has been developed from CdS QDs to  $\text{Ru}(\text{bpy})_3^{2+}$ . By the signal amplification of  $\text{Ru}(\text{bpy})_3^{2+}$  and the specific antibody-cell surface interactions, this ECL-RET system can sensitively respond down to  $12.5 \text{ SMMC-7,721 cells mL}^{-1}$  [75]. In the same group, an ultrasensitive DNA detection approach, which combines AuNPs enhanced ECL of the CdS nanocrystal film with isothermal circular amplification reaction of polymerase and nicking endonuclease, has been developed for the detection of DNA down to 5 aM [76].

### 4.3 Other Spectroscopic Measurements

Surface-enhanced Raman scattering (SERS)-based signal amplification and detection methods using plasmonic nanostructures have been widely investigated for imaging and sensing applications. A bifunctional adenosine-sensitive double-stranded DNA aptamer can create and control a SERS hot spot between a bulk Au surface and an AuNP attached to the aptamer via a biotin-avidin linkage. The AuNP is decorated with 4-aminobenzenethiol (4-ABT), a Raman reporter molecule. In the presence of adenosine, the target molecule, the SERS spectrum of 4-ABT increases in intensity by factors as large as ~4 [77]. In particular, SERS by molecules starts with an excitation, followed by inelastic coupling to internal vibrational levels of the molecule and a subsequent radiative decay, and therefore undergoing signal enhancement factors up to ten orders of magnitude by coupling



**Fig. 6** In situ inspection of pesticide residues on food/fruit. (a) Normal Raman spectra on fresh citrus fruits. Curve I, with clean pericarps; curve II, contaminated by parathion. Curve III, SHINERS spectrum of contaminated orange modified by Au/SiO<sub>2</sub> nanoparticles. Curve IV, Raman spectrum of solid methyl parathion. Laser power on the sample was 0.5 mW, and the collected times were 30 s. (b) Schematic of the SHINERS experiment. Reprinted with permission from Li et al. [80]. © 2010, Nature

to plasmonic hot spots. Such a tremendous increase in SERS signal allows zeptomole detection [78]. A SERS-based single-molecule detection has also been reported by using gap-tunable gold–silver core–shell nanodumbbells. Using a stoichiometric control over the number of tethering DNA molecules on the AuNPs surface and a subsequent magnetic-particle-based separation method, Au nanoparticle heterodimers are successfully synthesized in a relatively high yield by means of a single-target-DNA hybridization [79].

A shell-isolated nanoparticle-enhanced Raman spectroscopy has been proposed for inspecting pesticide residues on food and fruit via Raman signal amplification by AuNPs with an ultrathin silica or alumina shell. Figure 6 shows that normal Raman spectra recorded on fresh orange with clean pericarps (curve I) or contaminated by parathion (curve II), which shows only two bands at about 1,155 and 1,525 cm<sup>-1</sup>, attributed to carotenoid molecules contained in citrus fruits.

By spreading shell-isolated NPs on the same surface, two bands can clearly be detected at 1,108 and 1,341 cm<sup>-1</sup> (curve III) that are the characteristic bands of parathion residues. The shell-isolated nanoparticle-enhanced Raman spectroscopy demonstrates tremendous scope as a simple-to-use, field-portable, and cost-effective analyzer [80].



Controlled assembly of gold nanorods induced by  $\text{Na}_3\text{PO}_4$  leads to a significant amplification of localized surface plasmon resonance (LSPR) signals. The strong affinity between Au and Hg alters the coupled LSPR signals due to the amalgamation of Hg and Au. This allows detection of Hg in aqueous solutions with ultrahigh sensitivity and excellent selectivity, without sample pretreatment [81]. A phase interrogation SPR system based on gold nanorod has led to a drastic sensitivity enhancement at a concentration as low as the femtomolar range for detecting antigen with more than 40-fold increase compared to the traditional SPR biosensing technique [82]. Two-photon Rayleigh scattering (TPRS) properties of gold nanorods can be used for rapid, highly sensitive, and selective detection of *Escherichia coli* bacteria from aqueous solution, without any amplification or enrichment in 50 colony forming units (cfu)  $\text{mL}^{-1}$  level with excellent discrimination against any other bacteria. TPRS intensity increases 40 times when anti-*E. coli* antibody-conjugated nanorods were mixed with various concentrations of *E. coli* O157:H7 bacterium [83].

## 5 Functional Nanomaterial-Amplified Electrochemical Detection

### 5.1 Enhanced Conductivity with Nanoparticles

Carbon-based nanomaterials show excellent conductivity to promote the direct electron transfer between the biomolecules and electrode surface [84]. Based on the excellent conductivity of SWNTs, a proof-of-principle of the terminal protection assay of small-molecule-linked DNA has been designed in quantitative analysis of the interaction of folate with a tumor biomarker folate receptor, and a detection limit of 3 pM folate receptor is achieved with desirable specificity and sensitivity [85]. Similarly, an electrochemical immunoassay strategy has been developed by using phospholipid-coated CNTs as the electrochemical labels. The quasilinear response is obtained in a logarithmic concentration scale within a four-order of magnitude concentration range from 5 pg  $\text{mL}^{-1}$  to 50 ng  $\text{mL}^{-1}$  with a readily achieved detection limit of 3 pg  $\text{mL}^{-1}$  [86].

Using SWNTs forest platforms with multi-label  $\text{Ab}_2$ -nanotube bioconjugates, a general amplification strategy has been designed for highly sensitive detection of a cancer biomarker in serum and tissue lysates. This approach provides a detection limit of 4 pg  $\text{mL}^{-1}$  (100 amol  $\text{mL}^{-1}$ ), for PSA in 10  $\mu\text{L}$  of undiluted calf serum, a mass detection limit of 40 fg [16]. A glucose oxidase-functionalized CNTs nanocomposite has been designed to label the signal antibodies for ultrasensitive multiplexed measurement of tumor markers using a disposable immunosensor array. The simultaneous multiplexed immunoassay method showed linear ranges of three orders of magnitude with the detection limits down to 1.4 and 2.2 pg  $\text{mL}^{-1}$  for CEA and AFP, respectively [87].

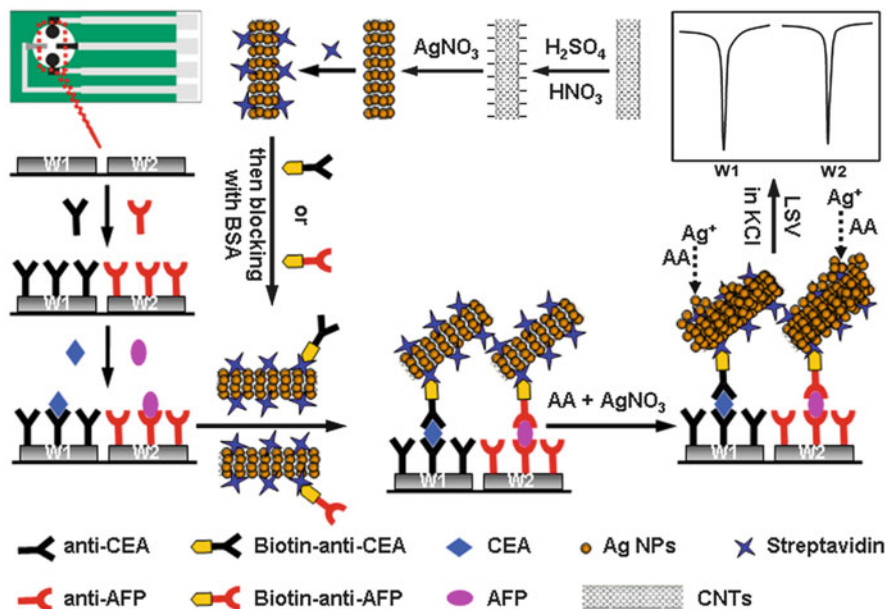
In cytosensing, RGDS-functionalized SWNTs are mainly used in two roles as nanoscaffolds for immobilization of cells and as nanoprobe to combine the specific recognition, signal transduction, and signal amplification abilities. The designed electrochemical cytosensor array has been used for simultaneous analyzing the dynamic change of the K562 cell-surface glycome during erythroid differentiation induced by sodium butyrate [88]. The result is consistent with the reference method.

AuNPs with quantum size effects and high electrical conductivity can accelerate electron transfer between redox enzymes and electrode surface. For example, when an apo-flavoenzyme, apo-glucose oxidase, is reconstituted on a 1.4-nanometer gold nanocrystal functionalized with the cofactor flavin adenine dinucleotide, the electron transfer turnover rate of the reconstituted bioelectrocatalyst ( $\sim 5,000 \text{ s}^{-1}$ ) is much larger than that of the natural cosubstrate of the enzyme ( $\sim 700 \text{ s}^{-1}$ ), providing an attractive route for electrochemical transduction of biorecognition events [89].

## 5.2 Direct Electrochemistry of Nanoparticle Aggregations

Utilizing the direct electrochemical signal of nanoparticle aggregations, many electrochemical assays have been developed for ultrasensitive detection. Typically, silver-enhanced labeling method is frequently employed in immunoassays for improving the sensitivity of detecting proteins. For example, an ultrasensitive multiplexed immunoassay has been developed by combining ALP-labeled antibody-functionalized AuNPs (ALP-Ab/AuNPs) catalyzed deposition of silver nanoparticles at a disposable immunosensor array. The deposited silver is then measured by anodic stripping analysis in KCl solution. This multiplexed immunoassay method shows wide linear ranges over four orders of magnitude with the detection limits down to 4.8 and  $6.1 \text{ pg mL}^{-1}$  for human and mouse IgG, respectively [90]. Subsequently, a streptavidin-functionalized silver-nanoparticle-enriched carbon nanotube (CNT/AgNP) is designed as trace tag for ultrasensitive multiplexed measurements of tumor markers (Fig. 7). The CNT/AgNP nanohybrid is prepared by one-pot in situ deposition of AgNPs on carboxylated CNTs. The nanohybrid is functionalized with streptavidin via the inherent interaction between the protein and AgNPs for further linkage of biotinylated signal antibodies to obtain tagged antibodies. Through a sandwich-type immunoreaction on the immunosensor array, numerous AgNPs are captured onto every single immunocomplex and are further amplified by a subsequent AgNP-promoted deposition of silver from a silver enhancer solution to obtain the sensitive electrochemical-stripping signal of the AgNPs. This multiplexed immunoassay method shows acceptable precision and wide linear ranges over four orders of magnitude with detection limits down to 0.093 and  $0.061 \text{ pg mL}^{-1}$  for CEA and AFP, respectively [91].

QDs exhibit sharp and well-resolved stripping voltammetry signals due to the well-defined oxidation potentials of the metal components. A CdTe QDs functionalized poly(styrene-co-acrylic acid) microbead as novel nanoparticle label has been used to amplify the electrochemical signal of DNA hybridization.



**Fig. 7** Schematic representation of preparation of immunosensor array and trace tag, and detection strategy by linear-sweep stripping voltammetric analysis of AgNPs on the immunosensor surface. Reprinted with permission from Lai et al. [91]. © 2011, Wiley

The mean quantum-dot coverage is  $(9.54 \pm 1.2) \times 10^3$  per polybead. By square-wave voltammetry of  $\text{Cd}^{2+}$  after the dissolution of the CdTe tags with  $\text{HNO}_3$ . The detection of the DNA hybridization process is achieved with a detection limit of  $0.52 \text{ fmol L}^{-1}$  and a dynamic range spanning five orders of magnitude [92]. Since the signals from multiple metal sulfide nanoparticles can be resolved by anodic stripping voltammetry, a method can possibly be extended to detect a multitude of hybridization events with 100 aM sensitivity by detecting the amplified electrochemical signal [93].

Further, combining the rolling circle amplification technique with oligonucleotide-functionalized QDs, a cascade signal amplification strategy has been proposed for detection of protein target at ultralow concentration. The designed strategy can quantitatively detect protein down to 16 molecules in a 100  $\mu\text{L}$  sample with a linear calibration range from 1 aM to 1 pM and is amenable to quantification of protein target in complex biological matrixes [94].

A sensitive electrochemical aptasensor for the detection of thrombin has been prepared by the amplification of nanoparticles and the usage of differential pulse voltammetry for the detection of dissolved  $\text{Cd}^{2+}$  in the solution. This assay can directly detect thrombin with a low detection limit of 0.55 fM [95]. Coupling of aptamers with the coding and amplification features of multiple metal sulfide nanoparticles, a highly sensitive and selective panel has been designed for simultaneous detection of several protein targets [96]. By measurement of  $\text{Au}^{3+}$  ions,

a simple electrochemical DNA probe based on submicrometer-size latex spheres with AuNPs is developed for detection of DNA hybridization with a detection limit of 0.5 fM [97].

The ECL emission of QDs is another way to sensitively detect target DNA concentration and sequence by using QDs as the tags. A sensitive DNA ECL sensor based on both the quenching and enhancement of ECL from CdS:Mn NCs by AuNPs in one assay has been constructed. The favorable response of 50 aM target DNA indicates a remarkably low detection limit. Such energy transfer in ECL systems opens a new way for transduction of biological recognition events [98].

Recently, photoelectrochemical assays based on the functional NPs have been quickly developed. These techniques hold the advantages of both optical and electrochemical detections. A photoelectrochemical biosensing platform has been constructed for the detection of biomolecules at relatively low applied potentials using porphyrin-functionalized TiO<sub>2</sub> nanoparticles. The proposed photoelectrochemical method can detect glutathione ranging from 0.05 to 2.4 mmol L<sup>-1</sup> with a detection limit of 0.03 mmol L<sup>-1</sup> at a signal-to-noise ratio of 3 [99]. Similarly, a photoelectrochemical platform based on free-base-porphyrin-functionalized Zinc oxide nanoparticles is developed for photoelectrochemical detection of cysteine with a linear range of 0.6–157 mmol L<sup>-1</sup> in physiological media [100].

### 5.3 *Electrocatalysis of Nanoparticles*

It is the alternative way to realize the ultrasensitive detection based on electrocatalysis of nanoparticle. Typically, an ultrasensitive and simple electrochemical method for signal amplification is achieved by catalytic reduction of *p*-nitrophenol to *p*-aminophenol using gold-nanocatalyst labels. The detection limit of this assay is 1 fg mL<sup>-1</sup> for mouse IgG corresponding to ca. 7 aM. This assay also achieves 1 fg mL<sup>-1</sup> detection limit for PSA, which is comparable to that of the bio-barcode assay [101].

A sandwich-type DNA sensor has been proposed by employing PdNPs as electrocatalytic label. To achieve low level of nonspecific binding of DNA-conjugated PdNPs, indium–tin oxide (ITO) electrode is firstly modified with a silane copolymer containing poly(ethylene glycol) and carboxylic acid. Then, amine-terminated capture probe is covalently attached to a silane copolymer-modified ITO electrode. After target DNA is hybridized with capture probe on the electrode, the fast catalytic hydrolysis of NaBH<sub>4</sub> on PdNPs generates many atomic hydrogens, which are rapidly absorbed into Pd NPs, leading to the rapid enhancement of electrocatalytic activity of Pd NPs. PdNP-based ultrasensitive DNA sensor shows an ultralow detection limit (10 aM) and a wide detection range (ten orders of magnitude) [102].

Fe<sub>3</sub>O<sub>4</sub> magnetic NPs are highly effective as a catalyst, with a higher binding affinity for the substrate 3,3',5,5'-tetramethylbenzidine than HRP and a 40-fold higher level of activity at the same molar concentration of catalyst [103]. A simple

and sensitive biosensor has been developed for the detection of DNA hybridization based on flow injection-CL and signal amplification by bio-bar-code functionalized magnetic nanoparticle labels, in which a large amount of metal ions are released from the magnetic NPs. Thus, an ultrasensitive detection of DNA hybridization is achieved by the luminol- $\text{H}_2\text{O}_2$ - $\text{Fe}^{3+}$  CL system with the detection limit as low as 0.32 fM without any preconcentration process [104].

The amplification strategy based on platinum nanoparticles (PtNPs) catalyzing a hydrogen evolution reaction has been developed for the ultrasensitive electrochemical immunosensing. After a typical immuno-sandwich protocol, the signal readout is obtained electrochemically via a PtNPs-catalyzed hydrogen evolution reaction in an acidic aqueous medium containing 10 mM of HCl and 1 M of KCl [105]. A multiple amplification immunoassay has also been proposed to detect AFP, which is based on ferrocenemonocarboxylic-HRP conjugated on PtNPs as labels for rolling circle amplification. The enzymatic amplification signal can be produced by the catalysis of HRP and PtNPs with the addition of  $\text{H}_2\text{O}_2$ , resulting in high sensitivity of the immunoassay with the detection limit of  $1.7 \text{ pg mL}^{-1}$  [106].

## 6 Conclusions and Perspectives

Based on the unique properties of nanomaterials, a wide variety of nanoscaled materials with different sizes, shapes, and compositions have been introduced into biosensing for signal amplification. The nanoparticles can be functionalized with biomolecules via noncovalent interaction and covalent route for specific recognition. The biofunctional nanoparticles can produce a synergic effect among catalytic activity, conductivity, and biocompatibility. Therefore, the biofunctional NPs have been used as carriers or tracers for design of a new generation of electronic, optical, and photoelectrochemical biosensing devices. Many considerations such as the good biocompatibility, the sufficient binding sites for functionalization, capacity in the multiple analysis, and so on should be emphasized in the development of ultrasensitive bioassay based on the biofunctional nanomaterials systems. In addition, the photoelectrochemical assays, which hold the advantages of both optical and electrochemical detections, should be a promising direction for constructing a ultrasensitive tool. Signal amplification strategies based on nanomaterials not only provide an ultrasensitive assay in detection of trace analytes but also a concept for basic research in nanobiosensing.

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Applications of Nanomaterials in Sensors and  
Diagnostics

Tuantranont, A. (Ed.)

2013, X, 285 p., Hardcover

ISBN: 978-3-642-36024-4