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TUTORIAL REVIEW

Signal amplification using functional nanomaterials for biosensing

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Signal amplification based on biofunctional nanomaterials has recently attracted considerable attention due to the need for ultrasensitive bioassays and the trend towards miniaturized assays. The biofunctional nanomaterials can not only produce a synergic effect among catalytic activity, conductivity and biocompatibility to accelerate the signal transduction, but also provide amplified recognition events by high loading of signal tags, leading to a highly sensitive and specific biosensing. Most importantly, nanoscaled materials are in direct contact with the environment, which permits them to act as chemical and biological sensors in single-molecule detection of biomolecules. In this *tutorial review*, we will focus on recent significant advances in signal amplification strategies combining the cross-disciplines of chemistry, biology, and materials science, and highlight some elegant applications of biofunctional nanomaterials as excellent electronic or optical signal tags in ultrasensitive bioanalysis. The biofunctional nanomaterials-based biosensing opens a series of concepts for basic research and offers new tools for detection of trace amounts of a wide variety of analytes in clinical, environmental, and industrial applications.

1. Introduction

The ultrasensitive detection of biomolecules is required in a variety of societal areas including clinical diagnostics, food safety, and environmental protection. Many efforts have been devoted to realizing the ultrasensitive and even single-molecule

detection by using signal amplification such as polymerase chain reaction and mass spectrometric techniques.^{1,2} Although these methods have adequate sensitivity, they are destructive and often suffer from time-consuming derivatization, high cost and professional operation. With the achievements of nanotechnology and nanoscience, nanomaterial-based signal amplifications hold great promise in realizing high sensitivity and selectivity for *in situ* or online detection of biomolecules due to the rapid analysis procedure and easy miniaturization.

Biofunctional nanoparticles (NPs) can produce a synergic effect among catalytic activity, conductivity and biocompatibility

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to accelerate the signal transduction, leading to lower and lower detection limits, even reaching zeptomolar concentrations. Furthermore, the linear correlation concentration range also becomes wider and wider, even 6–10 orders of magnitude. For example, an electrochemical DNA assay method exhibits an electrochemical signal from multiple metal sulfide NPs.³ Using superparamagnetic particle labels for signal amplification, a surface plasmon resonance (SPR) immunoassay method can detect a cancer biomarker, serum prostate specific antigen (PSA), at an ultralow detection limit (DL) of 10 fg mL^{-1} .⁴ More importantly, nanoscaled materials are in direct contact with the environment, which permits them to act as chemical and biological sensors in single-molecule detection of biomolecules.

In order to obtain the excellent selectivity and high sensitivity of biosensing, a suitable method should be introduced for the functionalization of nanomaterials with biomolecules as specific recognition or/and signal triggering elements. The approaches for the functionalization of NPs usually include: noncovalent interaction such as physical adsorption, specific affinity interaction and entrapment of biomolecules around the NPs, and covalent interaction of biomolecules with the functional groups on the NP surface.⁵ In signal amplification strategies, the nanomaterials usually act as catalysts to trigger the detectable signal or carriers for high loading of signal tags.

The noncovalent approach *via* electrostatic interaction, π – π stacking, or Van der Waals forces is an efficient immobilization method for biomolecules, which can avoid the destruction of conjugated skeletons and loss of electronic properties of the NPs. Covalent binding of the functional biomolecules to NPs should be preferable to unspecific physisorption in terms of stability and reproducibility of the surface functionalization. It can be performed by direct chemical reaction, linker chemical reaction or click chemistry. In addition, some affinity interactions such as avidin–biotin and barnase–barstar systems are also effective for robust assembly of multifunctional superstructures. The resulting conjugations of NPs with biomolecules can be endowed with the abilities of both recognition of target molecules and signal amplification. They have been extensively employed for excellent signal transduction of biological phenomena in development of electronic, optical, and photoelectrochemical biosensors (Fig. 1).

The optical assay is advantageous in biosensor design because of its high sensitivity, wide dynamic range, and multiplexing capabilities. In comparison with organic dyes and fluorescent proteins, optical nanoparticle probes such as fluorescence energy transfer nanobeads and quantum dots (QDs) provide significant advantages in signal brightness, photostability, and multicolor-light emission. These nanoprobe have been coupled with fluorescent, surface-enhanced Raman scattering and Rayleigh scattering detection, and even fluorescent and photoacoustic imaging. Recently the functional nanomaterials have attracted considerable interest in colorimetric, chemiluminescent, and visual detection of bioanalytes.

Electrochemical assays of bioanalytes based on the nanoprobe are attractive because of their low cost, high sensitivity, simplicity and easy miniaturization. The signal amplification strategies based on the catalytic ability or/and high conductivity of nanoprobe and the electrochemical stripping analysis of nanoprobe have been quickly developed due to the high sensitivity.

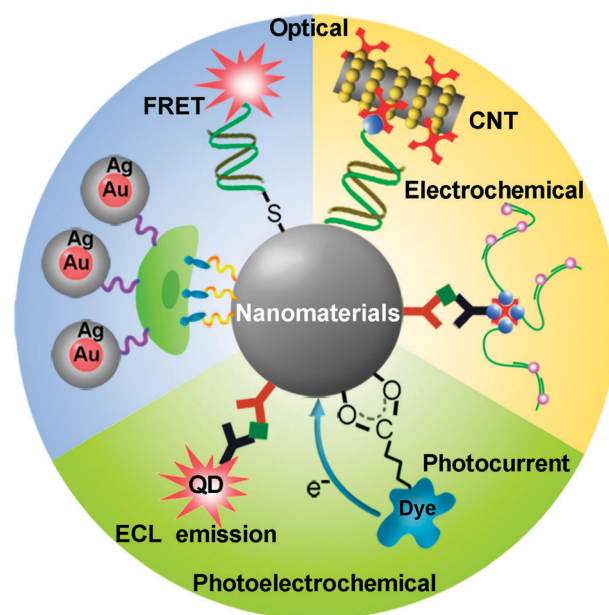


Fig. 1 Schematic illustration of signal amplification strategies using biofunctional nanomaterials in biosensing.

The electrochemiluminescent (ECL) and photoelectrochemical assays possess the advantages of both optical and electrochemical detections, and are also a promising perspective.

In this tutorial review, we highlight the recent significant advances in nanomaterial-based signal amplification strategies including the novel functionalization methods for the efficient immobilization of biomolecules as recognition or/and signal triggering elements on nanomaterials, and the functions of nanomaterials in signal amplification, and finally summarize some elegant applications of biofunctional nanomaterials as signal tags in ultrasensitive bioanalysis and biosensing.

2. Biofunctionalization methods for nanomaterials

2.1 Noncovalent assembly of nanomaterials with biomolecules

To efficiently enhance the specificity and sensitivity of the detection signal, it is necessary to seek suitable methods for the functionalization of NPs with biomolecules as recognition and signal triggering elements. The noncovalent assembly of biomolecules on NPs has frequently been performed for biofunctionalization of NPs with biomolecules due to its nondestructivity. A general and attractive approach *via* π – π interactions has been designed by Dai and co-workers for the noncovalent functionalization of the sidewalls of single-walled carbon nanotubes (SWNTs) and the subsequent immobilization of biomolecules onto SWNTs *via* *N*-succinimidyl-1-pyrenebutanoate.⁶ The nonspecific binding of protein is further overcome by immobilization of polyethylene oxide chains on nanotubes, which enables highly specific electronic sensors for detection of the related substrates.

The electrostatic interaction is an alternative method to assemble biomolecules on the surface of NPs, particularly for deposition of macromolecules such as proteins or enzymes. Typically, gold nanoparticles (AuNPs) produced by citrate reduction can be multifunctionalized with antibody molecules

and enzymes at pH values that lie slightly above the isoelectric point of the citrate ligand, which offers multienzyme tags for each antibody–antigen binding event to produce signal amplification. Similarly, since the carboxylate group decorated QDs are negatively charged in neutral or basic buffers, the positively charged molecules such as cationic polymer can be used for electrostatic functionalization of QDs. The polymer can then be further functionalized by layer-by-layer assembly.

Another noncovalent method for immobilizing biomolecules on NPs is to entrap them in biocompatible films such as polymer, Nafion, chitosan and DNA. The coating films not only prevent the aggregation of NPs but also provide abundant positions for functionalization of NPs with the second biomolecules. Especially, an electroactive polymer which can generate a large number of electrons during electrochemical oxidation to amplify the electrochemical signal and therefore enhance the detection sensitivity. Single strand DNA (ssDNA) can wrap around SWNTs through aromatic interaction to form a soluble DNA–SWNTs complex, which is promising for construction of a highly sensitive biosensor for target biomolecules.⁷

2.2 Covalent route for biofunctionalization of nanomaterials

Compared to noncovalent methodologies, the formation of NP conjugates employing covalent methods bears a number of advantages. One is that the controllable chemisorption *via* covalent binding in general should be preferable to unspecific physisorption in terms of stability and reproducibility of the surface functionalization. The other is that the number of functional groups are controllable by fine-tuning the functionalization processes. Based on the different types of chemical reactions, the covalent binding modes can be divided into three strategies.

In the direct reaction strategy, functional groups at the NP surfaces can be directly bound to reactive ligands by a linkage reaction facilitated with the aid of catalysts. Typically, carbon nanotubes (CNTs) can be firstly shortened by sonication in 3:1 H₂SO₄/HNO₃ 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.⁸ This approach has been used in the attachment of DNA, aptamer and antibody–antigen to NPs. Similarly, amino-decorated NPs can conjugate with biomolecules bearing carboxylated groups for the attachment of peptides, proteins, antibodies, and enzymes to NPs. As to the metal NPs, the primary binding of thiolated molecules, such as thiolated oligopeptides, to NPs can provide a means for the covalent tethering of biomolecules to NPs.⁹ In some cases, tight chemisorption of proteins on metal NPs can originate from the binding of amino or thiol groups existing in the proteins to the NP surface. For metal oxide NPs, the binding of carboxylate ligands and phosphonate groups of biomolecules to their surface *via* monodentate coordination with bridging chemisorption or chelating chemisorption is one useful way.⁵

Biomolecules in direct contact with an unprotected solid substrate is subject to more or less severe denaturing and the ensuing loss of their specific biochemical functionality. Thus, low-molecular bifunctional linkers, which have anchor groups for their attachment to NP surfaces and functional groups for

their further covalent coupling to the target biomolecules, have been extensively used in the generation of covalent-tethered conjugates of biomolecules with various NPs. The most common amine, active ester, and maleimide groups are used to couple biological compounds covalently by means of carbodiimide-mediated esterification and amidation reactions or through reactions with thiol groups.⁵ The main role of the linker molecules is to not only provide a high density of docking sites for the specific attachment of biomolecules but also maintain a sufficiently low density of electronic defects at the NP surface.

To design a modular and broadly applicable targeting platform, Weissleder and co-workers¹⁰ described a covalent, bioorthogonal reaction between a 1,2,4,5-tetrazine and a *trans*-cyclooctene for small molecule labelling (Fig. 2a). The [4 + 2] cycloaddition is fast, chemoselective, does not require a catalyst, and can be adapted to targeting NP sensors in different configurations to improve binding efficiency and detection sensitivity. They comparatively tested the bioorthogonal NP detection (BOND) technique for targeting extracellular receptors on cancer cells using two assay types: directly coupled magneto-fluorescent nanoparticles (MFNPs) to antibodies before cell exposure (BOND-1) and a two-step strategy (BOND-2) (Fig. 2b). BOND-2 resulted in significantly higher nanoparticle binding, exceeding that of BOND-1 by a factor of 15. The mild strategy *via* 1,3-dipolar cycloaddition was also applied in the covalent functionalization of radionuclide-filled SWNTs as radioprobes.¹¹

“Click” chemistry, a Cu-catalyzed azide–alkyne cycloaddition, is a relatively new approach for easy and almost quantitative functionalization, developed by Sharpless *et al.* almost a decade ago. Click reactions on NP surfaces can be performed by firstly decorating the surface with either alkyne or azide and then conjugating to biomolecules. These reactions are fast and efficient. They require mild reaction conditions (aqueous environment, relatively neutral pH), and create water-soluble

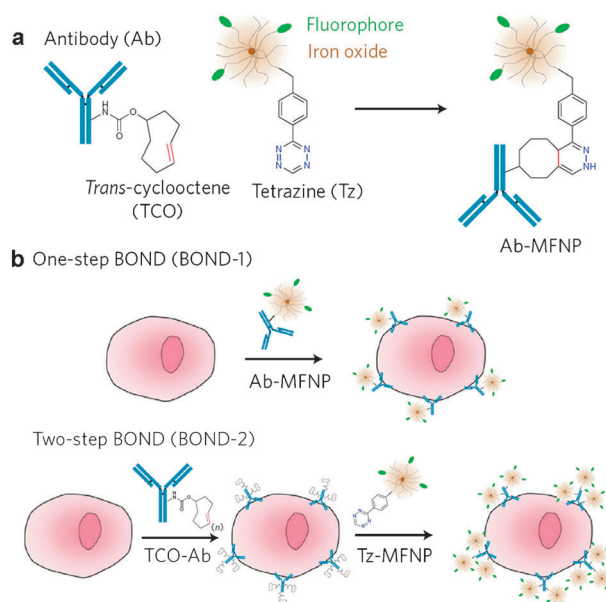


Fig. 2 (a) Schematic presentation of the conjugation chemistry between antibody and nanoparticle. (b) Application of bioorthogonal NP for one-step (direct) and two-step targeting of NPs to cells [reprinted with permission from ref. 10].

Table 1 Signal amplification using biofunctional nanomaterials in biosensing

Nanohybrid	Functionalization method	Type of detection signal	Analyte	Linear range/DL	Ref.
CNT/HRP	Direct covalent interaction	DPV of enzymatically liberated 2,2'-diaminoazobenzene	DNA	10 aM–1 pM/2.8 aM	17
CNT/ALP	Indirect covalent interactions	Chronopotentiometry of the enzymatically liberated α -naphthol	DNA	0.01–100 pg mL ⁻¹ / 1 fg mL ⁻¹	18
CNT/DNA	Direct covalent interaction	Field-effect transistor	DNA	Single-molecule detection	19
SWNT/DNA	Noncovalent interaction	Near-infrared emission of SWNT	H ₂ O ₂	Single-molecule detection	20
SWNT/ssDNA	π - π interaction	Direct electrochemistry of guanine	DNA	40–110 nM/20 nM	7
SWNT/RGDS	Direct covalent interaction	DPV of enzymatically liberated 2,2'-diaminoazobenzene	Cell	1.0×10^3 to 1.0×10^7 cells mL ⁻¹ /620 cells mL ⁻¹	21
CNSs/HRP–Ab ₂	Direct covalent interaction	DPV of enzymatically liberated 2,2'-diaminoazobenzene	AFP	0.05–6 ng mL ⁻¹ / 0.02 ng mL ⁻¹	22
Graphene/TMPyP	π - π interaction	UV-visible spectroscopy	Cd ²⁺ ions	0.2–1 μ M/N.A. ^a	23
AuNPs/aptamer	Au–S chemistry	Colorimetric biosensor based on AuNP aggregation	Adenosine	20–2000 μ M/N.A.	24
AuNPs/DNA–HRP	Direct covalent interaction	Colorimetric analysis	DNA	25 pM–1.0 nM/1 pM	9
AuNPs/DNA–peptide	Au–S chemistry	Fluorescence signal	MMP-2 ^b	N.A./10 pM	25
Au/SiO ₂ nanoparticles	Coating SiO ₂ onto AuNPs	SERS	Methyl parathion	N.A.	26
Au–MWNTs/anti-MC-LR	Direct covalent interaction	DPV of enzymatically liberated 2,2'-diaminoazobenzene	MC-LR	0.005–1.0 mg L ⁻¹ / 0.002 mg L ⁻¹	27
Au–IgG	Direct covalent interaction	CV of ferrocene oxidation amplified by chemical reduction of <i>p</i> -quinone imine to AP by NaBH ₄	Mouse IgG	1 fg mL ⁻¹ –10 μ g mL ⁻¹ / 1 fg mL ⁻¹	28
Pd NPs/DNA	Pd–S	Electrocatalytic oxidation of sodium borohydride	DNA	10 aM–100 nM/10 aM	29
Ag NPs/CNT	Noncovalent interaction	Direct electrochemistry of Ag NPs	CEA	0.1 pg mL ⁻¹ –5.0 ng mL ⁻¹ / 0.093 pg mL ⁻¹	30
CdSe–ZnS QDs/aptamer	Direct covalent interaction	Chemiluminescence resonance energy transfer	Hg ²⁺	0.025–1 μ M/10 nM	31
Streptavidin–QD/DNA	Streptavidin–biotin	FRET from QDs to Cy5	DNA	N.A.	14
Streptavidin/QD	Streptavidin–biotin	FRET	DNA methylation	N.A./15 pg	32
CdSe/DNA	Direct covalent interaction	Fluorescence of Rhod-5N molecules	miR21	N.A./35 fM	33
CdS-, ZnS-, and PbS/DNA	Metal–S	DPV of QDs	DNA	N.A./0.1 fM	3
CdTe QDs/Con A	Covalent route	Anodic stripping signal of QDs	Cell surface carbohydrate	2.3×10^{10} mannose moieties per single K562 cell	15
MB/streptavidin–Ab ₂	Streptavidin–biotin	Amperometric response	Interleukin 8	1–500 fg mL ⁻¹ /1 fg mL ⁻¹	34
Tosyl-activated magnetic bead/Ab ₂	Covalent route	SPR immunosensor	PSA	N.A./10 fg mL ⁻¹	4
Fe ₃ O ₄ /DNA	Nucleic acid–DNA	Chemiluminescence of luminol	DNA	N.A./10 fM	16
Streptavidin/magnetic nanotag	Coating streptavidin on magnetic nanotag	Spin valve sensor	hCG ^c	N.A./subpicomole	35

^a N.A.: not available. ^b MMP-2: matrix metalloproteinase-2. ^c hCG: human chorionic gonadotropin.

and biocompatible linkages. Compared to other direct conjugation strategies, this attachment method offers several unique features with high specificity, high stability and extreme rigidity. *Via* the one-step click reaction, drug-loaded polymer NPs can be functionalized with folate, biotin, and gold NPs for drug delivery.¹² A gradient of a glycine-arginine-glycine-aspartate-serine linear peptide has been fabricated on a versatile substrate by using click chemistry technology as a tool for screening surface-directed cell function.¹³

2.3 Specific affinity interaction between nanomaterials and biomolecules

Affinity interaction is very effective for bioconjugation of targeting ligands to NPs due to the specific and strong

complementary recognition interactions such as antigen–antibody, nucleic acid–DNA, lectin–glycan, streptavidin–biotin, aptamer–protein, aptamer–small biomolecule and hormone–receptor interactions. The linkage formed is highly stable and the strongest in all non-covalent linkages. Moreover, most biomolecules contain several binding sites, for example, antibodies exhibit two Fab (antigen-binding fragment) sites, and streptavidin or concanavalin A each displays four binding domains. This allows the multidirectional growth of NP structures.^{14,15} The aptamer functionalized NPs are a promising platform for constructing new biosensing and bioanalytical systems by the specific recognition of aptamer toward target biomolecules.

Different from the avidin–biotin system and complementary DNA assembled scaffolds, a new generic method based on the barnase and barstar system has been employed for robust

self-assembly of multifunctional superstructures consisting of different single-function particles as labels, carriers, recognition, and targeting agents, *etc.*¹⁶ The link between the particles is very specific and strong enough to hold macroscopic (5 nm–3 μm) particles together. This method can be used to join inorganic moieties, organic particles, and single biomolecules for synergistic use in different applications such as biosensors, photonics, and nanomedicine.

3. Strategies of signal amplification

A lot of nanomaterials, such as metal NPs, semiconductor NPs and carbon nanostructures, have been introduced for the biofunctionalization *via* physical adsorption, electrostatic binding, specific recognition or covalent coupling. These biofunctionalized nanomaterials can be used as carriers or tracers, catalysts, electronic conductors, and optical emitters to obtain the amplified detection signal and the stabilized recognition probes. Combining with biotechnology, the detection signal based on nanomaterials amplification can be further enhanced. The designed signal amplification strategies have extensively been used in the development of sensitive biosensing devices, including optical and electronic biosensors (Table 1).

3.1 Nanomaterials as carriers for signal amplification

Due to their unique properties, NPs, especially carbon and metal-based NPs, are excellent candidates as carriers to enhance the probe area with their good conductivity and biocompatibility. The NPs can be employed as supporters at the bottom for concentrating the capture molecules and/or largely loading signal molecules. Typically, SWNTs have been used to immobilize the probe DNA for fabricating a DNA hybridization biosensor. Based on the direct current response of guanine, the response was proportional to the concentration of target DNA in the range of 40–110 nM with a detection limit of 20 nM. Ready renewal is the outstanding merit of this label-free biosensor, which can be reused more than 3000 times.⁷

Compared with SWNTs, nitrogen-doped carbon nanotubes (CNx-MWNTs) have lower cytotoxicity and better biocompatibility, making them suitable for loading biomolecules to construct biosensors. Ju and co-workers²⁷ developed a

convenient and green chemical route for directly attaching AuNPs onto CNx-MWNTs, and used the resulting Au/CNx-MWNTs nanocomposite as an immobilization scaffold for antibody for preparation of a sensitive immunosensor to detect microcystin-LR (MC-LR). Under optimal conditions, the immunosensor exhibited a wide 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. The detection limit was much lower than the limit set out in the WHO guidelines.

Functionalized graphene sheets have been used as a biosensor platform due to their increased surface area to capture a large amount of protein, thus amplifying the detection response. On the basis of the dual signal amplification strategy of graphene sheets and the multienzyme labeling on carbon nanospheres (CNSs), an immunosensor showed a 7-fold increase in detection signal compared to the immunosensor without graphene modification and CNSs labeling. The proposed method could respond to 0.02 ng mL^{-1} α -fetoprotein (AFP) with a linear calibration range from 0.05 to 6 ng mL^{-1} .²² Based on the supramolecular assembly of free-base cationic 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin (TMPyP) on reduced graphene, the resulting graphene–porphyrin hybrid as an optical probe has been constructed for rapid and selective sensing of Cd^{2+} ions in aqueous media.²³

Magnetic NPs easily achieve concentration and purification of analysts, which is useful to dramatically enhance the sensitivity in biosensing. Drndić and co-workers³⁶ developed a protocol for nanopore-based microRNA (miRNA) detection as shown in Fig. 3. In step (I), the probe:miRNA duplex is enriched by binding to p19-functionalized magnetic beads, followed by thorough washing to remove other RNAs from the mixture. In step (II), the hybridized probe:miRNA duplex is eluted from the magnetic beads. In step (III), the eluted probe:miRNA duplex is electronically detected using a nanopore. Using p19-functionalized magnetic beads, over 100 000-fold enrichment of the probe:miRNA duplex was achieved from total RNA. This approach was validated by detecting picogram levels of a liver-specific miRNA (miR122a) from rat liver RNA.

AuNPs have been also used as carriers of the signaling molecules for amplification detection of DNA and protein targets. The AuNPs modified telomeric repeat amplification

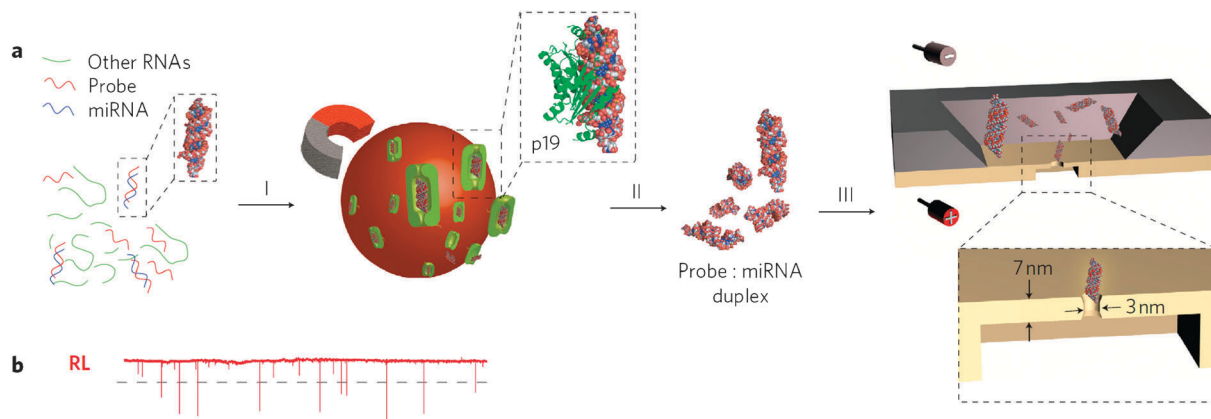


Fig. 3 miRNA detection based on the enrichment *via* p19-functionalized magnetic beads using solid-state molecular counters. (a) Scheme of the miRNA-specific detection method. (b) Detection of miR122a from 1 mg of rat liver total RNA using a 3-nm-diameter nanopore in a 7-nm-thick membrane [reprinted with permission from ref. 36].

protocol can achieve exceptional sensitivity and selectivity, detecting as few as 5 cancer cells even when they are doped with up to 5000 telomerase-free somatic cells.³⁷ A homogeneous colorimetric DNA biosensor has been developed by a novel nicking endonuclease assisted AuNPs amplification, resulting in a 10^3 -fold improvement in amplification (*ca.* 10 pm) and the capability of recognizing long single-stranded oligonucleotides with single-base mismatch selectivity.³⁸ Using small molecule self-assembled monolayers (SAMs) on AuNPs as a reporter, a new signal amplification strategy has realized attomolar detection of target proteins by combining with laser desorption/ionization mass spectrometry with a matrix-free format.²

Due to the small size, high surface-to-volume ratio and good biocompatibility, silica nanoparticles have become another normally used carrier for signal amplification. An organically capped mesoporous inorganic material has been employed for selective CH_3Hg^+ determination through loading large amounts of dye molecules [safranin O] for signal amplification. The use of lipophilicity partitioning guaranteed the required selectivity for CH_3Hg^+ , which has not yet been accomplished for other low-cost and time-effective sensory methods, whilst reaching comparable sensitivity in the ppb range.³⁹ This promising proof-of-principle has successfully been applied in the detection of methylmercury in fish samples.

3.2 Nanomaterials-amplified optical assay

Optical detection is advantageous in biosensor design because of its high sensitivity, wide dynamic range, and multiplexing capabilities. Moreover, NP probes provide significant advantages in signal brightness, photostability, and multicolor-light emission. Metal NP-based homogeneous colorimetric detection of oligonucleotides holds great promise due to low-cost, low-volume, and rapid readout of a target DNA sequence. For example, colorimetric detection of DNA sequences based on electrostatic interactions with unmodified AuNPs can be completed within 5 min, and <100 femtomoles of target produces color changes observable without instrumentation.⁴⁰ The colorimetric barcode assay based on AuNPs also offers both the attomolar sensitivity and the simple, portable and low cost DNA detection method without instrumentation.

The use of functional AuNP development has led to a series of amplified optical assay methods such as surface-enhanced Raman scattering and Rayleigh scattering analysis and visual detection of bioanalytes. The greater signal enhancement can be obtained by catalytic deposition of gold or silver NPs, which can increase the light scattering signal of the immobilized AuNP probes. The visual signal can be obtained by scanometric detection schemes.

Fan and co-workers⁹ developed a “sandwich-type” detection strategy, in which biotinylated capture probes were loaded on avidin-modified magnetic microparticles while thiolated detection probes were assembled on AuNPs *via* Au–S bonds. In the presence of target DNA, the capture probe recognized the target DNA, along with the detection probe, due to the proximity of magnetic microparticles, and this complex was then magnetically separated for subsequent optical detection. This method could conveniently detect as few as 100 pM target DNA with the naked eye. Furthermore, this sensitivity could be significantly improved

by instrument-based assays. 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.²⁴

Based on the fluorescence signal recovery after digestion of RNA by RNase H, Chung and Kim²⁵ reported a strategy of fluorescence signal amplification for highly sensitive and rapid protease assay. This method permitted the assessment of the activity of matrix metalloproteinase-2 at concentrations as low as 10 pM within 4 h. Compared with the previously reported protease nanosensors, the assay time of this method was six-fold shorter and the detection limit was 100-fold more sensitive.

In the presence of Cu^{II} with sodium ascorbate as the reductant, AuNPs that have azide- and alkyne-terminated groups undergo aggregation as the result of Cu^{I} -catalyzed click chemistry. Therefore, a colorimetric immunoassay based on the detection of Cu^{II} released from copper monoxide nanoparticle-labeled antibodies as the secondary antibody (Ab_2) has been developed for the detection of human immunodeficiency virus, in place of the fluorescent dye- or enzyme-labeled Ab_2 traditionally used in immunoassays.⁴¹ At ambient temperature, this process can be monitored by the naked eye. This method is highly specific even in the presence of high concentrations of mixtures of other cations and interfering molecules.

A shell-isolated NP-enhanced Raman spectroscopic method has been proposed for inspecting pesticide residues on food and fruit *via* Raman signal amplification by AuNPs with an ultrathin silica or alumina shell. The clean pericarps show only two bands at about 1155 cm^{-1} and 1525 cm^{-1} , attributed to carotenoid molecules contained in citrus fruits. By spreading shell-isolated NPs on the same surface, two bands can clearly be detected at 1108 cm^{-1} and 1341 cm^{-1} that are characteristic bands of parathion residues. The shell-isolated NP-enhanced Raman spectroscopy demonstrates tremendous scope as a simple-to-use, field-portable and cost-effective analyzer.²⁶

Semiconductor nanoparticles have attracted substantial research interest as fluorescence labels for biorecognition processes due to size dependent tunable absorbance and fluorescence. Based on fluorescence resonance energy transfer (FRET), an ultrasensitive and reliable nanotechnology assay is set up for detection and quantification of DNA methylation. This approach can detect as little as 15 pg of methylated DNA in the presence of a 10000-fold excess of unmethylated alleles, and allows for multiplexed analyses.³² In addition, a much simpler and milder strategy amplifies fluorescence signals by the cation-exchange reaction with ionic nanocrystals. The 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 in protein detection 100 times lower than that of the organic fluorophore Alexa 488.⁴²

More recently, Willner and co-workers³¹ have implemented the DNazyme-stimulated chemiluminescence resonance energy transfer (CRET) to CdSe/ZnS QDs for developing aptamer or DNA sensing platforms. The sensitivity for the detection of the analyte DNA by the CRET process corresponds to 10 nM, which is comparable to other QDs-based FRET sensing platforms. In fact DNazyme or horseradish peroxidase functionalized NPs have widely been coupled with chemiluminescent systems to

trigger and enhance the chemiluminescent emission. When co-assembling these enzymes and antibody on the NP surface, the multifunctional NPs could be conveniently used for sensitive chemiluminescent immunoassays. Other biomolecules with recognition ability such as DNA, lectin, or aptamers can also be linked to the enzyme functionalized NPs for recognising the corresponding targets and developing chemiluminescent methods for sensitive quantitative detection of bioanalytes. Compared to horseradish peroxidase, DNAzyme has a smaller size and higher peroxidase-like activity, thus is a promising trigger in amplifying the chemiluminescent signal.

A biofunctionalized three-dimensional ordered nanoporous silica film has been used to construct a CL immunosensing device. The three-dimensional ordered nanopores have high capacity for loading of streptavidin and antibodies and improving the mass transport of immunoreagents in immunoreaction, thus the resulting CL immunosensor shows high sensitivity and a wide dynamic range for a fast immunoassay.⁴³

3.3 Nanomaterials-amplified electrochemical detection

Carbon-based nanomaterials and metal NPs show excellent conductivity to promote the direct electron transfer between the biomolecules and electrode surface. For example, SWNTs can act as a nanoconnector that electrically contacts the active site of the enzyme and the electrode.⁴⁴ The interfacial electron transfer rate constant of the enzyme at a 50-nm SWNT modified electrode is estimated to be 42 s^{-1} , which provides significant potential for constructing an electrochemical reagentless biosensor.

Based on the excellent conductivity of SWNTs, Jiang and co-workers⁴⁵ designed a proof-of-principle of the terminal protection assay of small-molecule-linked DNA (Fig. 4). In the absence of protein, the SWNT-wrapping ssDNA could be digested in a stepwise manner from the 3' end by Exo I, producing SWNTs with no surface-tethered ssDNA. These "naked" SWNTs were precipitated from the solution and assembled on the 16-mercaptohexadecanoic acid (MHA) SAM to mediate efficient electron transfer between the electrode and an electroactive species. Thus, a strong redox current was generated due to the signal amplification from a single SWNT to numerous electroactive molecules. In the presence of small-molecule-binding proteins, the SWNT wrapping ssDNA was bound to the protein target through the small molecule moiety at the 3' terminus, thus preventing the degradation of the ssDNA by Exo I. Therefore, the DNA-SWNT did not adsorb on the MHA SAM, resulting in no background current signal. This strategy was applied in quantitative analysis of the interaction of folate with a tumor biomarker of folate receptor, and a detection limit of 3 pM folate receptor was achieved with desirable specificity and sensitivity. 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 mL^{-1} to 50 ng mL^{-1} with a readily achieved detection limit of 3 pg mL^{-1} .⁴⁶

AuNPs with quantum size effects and high electrical conductivity can facilitate fast electron transfer to and from redox enzymes, providing a sensitive platform for biosensors. For example,

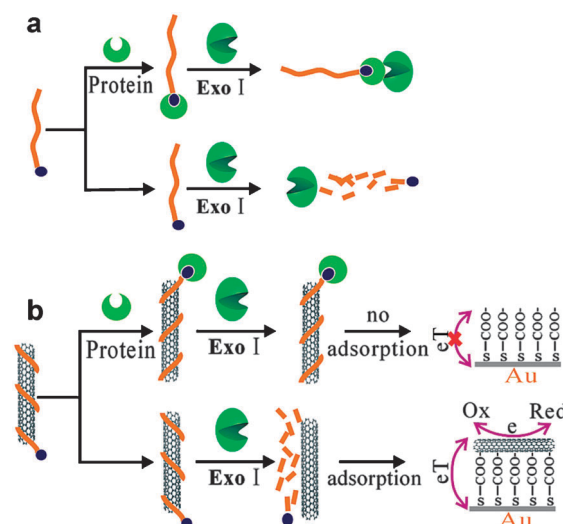


Fig. 4 Terminal protection assay of small-molecule-linked ssDNA. (a) Small-molecule-linked ssDNA is hydrolyzed successively into mononucleotides from the 3' end by Exo I, while protected from the hydrolysis when the small molecule moiety is bound to its protein target. (b) SWNT wrapping ssDNA terminally tethered to small molecule is degraded by Exo I, rendering SWNTs assembled on MHA SAM that mediates electron transfer between electroactive species and the electrode. Protein binding of small-molecule-linked ssDNA prevents digestion of ssDNA, precluding adsorption of DNA-wrapped SWNTs on MHA SAM with no redox current generation [reprinted with permission from ref. 45].

when an apo-flavoenzyme, apo-glucose oxidase, is reconstituted on a 1.4-nanometre gold nanocrystal functionalized with the cofactor flavin adenine dinucleotide, the electron transfer turnover rate of the reconstituted bioelectrocatalyst is $\sim 5000 \text{ s}^{-1}$, which is much larger than that of the natural cosubstrate of the enzyme (~ 700 per second). Thus this approach provides an attractive route for electrochemical transduction of biorecognition events.⁴⁷

Utilizing the electrochemical stripping analysis of NP aggregations, many analytical methods have been developed for ultrasensitive detection. These NP aggregations include AuNPs, AgNPs and QDs. A silver-enhanced labeling method is frequently employed in immunoassays for improving the sensitivity of detecting proteins. For example, an ultrasensitive multiplexed immunoassay has been proposed by using alkaline phosphatase (ALP)-labeled antibody functionalized AuNPs (ALP-Ab/AuNPs) to catalyze the deposition of silver NPs at a disposable immunosensor array, followed with anodic stripping analysis of the deposited silver in KCl solution. This multiplexed immunoassay method shows wide linear ranges over 4 orders of magnitude with the detection limits down to 4.8 and 6.1 pg mL^{-1} for human and mouse IgG, respectively.⁴⁸ Subsequently, a streptavidin-functionalized silver-nanoparticle-enriched carbon nanotube (CNT/Ag NP) is designed as a trace tag for ultrasensitive multiplexed measurements of tumor markers. Through a sandwich-type immunoreaction on the immunosensor array, numerous Ag NPs are captured onto every single immunocomplex and are further amplified by a subsequent Ag NP-promoted deposition of silver from a silver enhancer solution to obtain the sensitive electrochemical-stripping signal of the Ag NPs.

This proposed 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 pg mL^{-1} for carcinoembryonic antigen (CEA) and α -fetoprotein, respectively.³⁰

A CdTe QDs functionalized poly(styrene-*co*-acrylic acid) microbead as NP label is capable of amplifying the electrochemical signal of DNA hybridization. The mean quantum-dot coverage is $(9.54 \pm 1.2) \times 10^3$ per polybead. By square-wave voltammetric detection of Cd^{2+} after the dissolution of the CdTe tags with HNO_3 , the detection of the DNA hybridization process is achieved with a detection limit of 0.52 fmol L^{-1} and a dynamic range spanning 5 orders of magnitude.⁴⁹ 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. Using human vascular endothelial growth factor as a model protein, the designed strategy can quantitatively detect protein down to 16 molecules in a 100 μL sample with a linear calibration range from 1 μM to 1 pM . This method is amenable to quantification of protein target in complex biological matrices.⁵⁰

The electrocatalytic behavior of NPs or functionalized NPs is an alternative way to realize the ultrasensitive detection. Typically, a sandwich-type DNA sensor has been established by employing Pd NPs as electrocatalytic labels. The fast catalytic hydrolysis of NaBH_4 on Pd NPs generates many atomic hydrogens, which are rapidly sorbed into Pd NPs, leading to the rapid enhancement of electrocatalytic activity of Pd NPs. Because NaBH_4 undergoes multi-electron (maximum 8e^-) oxidation on Pd NPs, higher current signals can be obtained than those in the electrochemical reactions involving one-electron or two-electron oxidation. Pd NP-based ultrasensitive DNA sensor shows an ultralow detection limit (10 aM) and a wide detection range (10 orders of magnitude).²⁹

Magnetic nanoparticles (MNPs) possess an intrinsic enzyme mimetic activity similar to that found in natural peroxidases. Fe_3O_4 MNPs are highly effective as a catalyst, with a higher binding affinity for the substrate 3,3',5,5'-tetramethylbenzidine than horseradish peroxidase (HRP). At the same molar concentration, the Fe_3O_4 MNPs show an activity level 40 times higher than HRP. Based on this finding, a novel immunoassay has been developed, in which antibody-modified MNPs provide three functions: capture, separation and detection.⁵¹

Electrochemical impedance spectroscopic (EIS) technique has been used in functional NPs-based electrochemical detection. Upon the recognition of the bioanalyte by the functional NPs or biomolecules immobilized on the NP modified electrode surface, the electron transfer impedance of the redox probe increases, leading to a detectable signal for analysis of the bioanalyte. Here the NPs act as both carrier of the recognition species and/or the conductivity to promote the electron transfer between the redox probe and electrode surface. The latter decreases the background signal and thus improves the detection sensitivity. This technique is especially efficient for the detection of macrobiomolecules and living cells, thus has been used in cytosensing.

In addition, ECL and photoelectrochemical assays based on the functional NPs have been quickly developed in recent years. These techniques hold the advantages of both optical

and electrochemical detections. The general functional NPs used for ECL detection include II–VI, III–V and IV–VI nanocrystals (QDs), carbon NPs, and even aromatic hydrocarbon NPs, while functionalized ZnO and TiO_2 NPs have been used for photoelectrochemical biosensing. The functionalization of various QDs with multitudinous biomolecules has attracted considerable interest in finding new emitters with higher ECL efficiency and offering sensitive ECL signal transduction platforms for biosensing. Compared with the conventional organic emitters, QDs as ECL emitters show excellent advantages such as high fluorescence quantum yields, size or surface trap-control luminescence, and good stability against photobleaching. By combining the enzymatic cycle with the ECL process of QDs, some signal amplification strategies have been designed for ECL sensitive biosensing.

4. Single-molecule detection

The detection and identification of single molecules are considered to be the ultimate limit of sensitivity in chemical analysis and mechanistic biophysical studies. Nanoscaled materials have unique electronic and optical properties such as excellent conductivity, tunable light emission, signal brightness, and multicolor excitation that are not available from bulk materials. Moreover, the small sizes of nanoparticles break through the limitation of structure miniaturization, leading to the direct communication between nanoscaled materials and the analytes, which permits them to act as chemical and biological sensors in single-molecule detection of analytes.

Besides the sensitivity time resolution is also a technological requirement in exploring biomolecular systems with fast dynamics. 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 nanometre 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.⁵² Combining the localized surface plasmon resonance with an enzyme-linked immunosorbent assay, a simple colorimetric biosensing methodology with single molecule sensitivity has been developed by spectral imaging of a large number of isolated AuNPs.⁵³

FRET-based probes incorporated with single-molecule fluorescence detection technologies have allowed detection of DNA with low abundance without additional amplification. The QDs act as both a FRET energy donor and a target concentrator. The sandwiched hybrids are captured by a single QD through biotin–streptavidin binding. The resulting assembly brings the fluorophore acceptors and the QD donor into close proximity, leading to fluorescence emission from the acceptors by means of FRET on illumination of the donor. 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.¹⁴

Surface-enhanced Raman scattering (SERS)-based signal amplification and detection methods using plasmonic

nanostructures have been widely investigated for imaging and sensing applications. However, SERS-based molecule detection strategies have not been practically useful because there is no straightforward method to synthesize and characterize highly sensitive SERS-active nanostructures with sufficiently high yield and efficiency. Kim and co-workers⁵⁴ reported a SERS-based single-molecule detection by using gap-tailorable gold–silver core–shell nanodumbbells (Fig. 5). Using stoichiometric control over the number of tethering DNA molecules on the AuNPs surface and a subsequent magnetic-particle based separation method, AuNP heterodimers were successfully synthesized in a relatively high yield by means of a single-target-DNA hybridization. Atomic force microscope (AFM)-correlated nano-Raman measurements of individual dumbbell structures demonstrate that Raman signals can be repeatedly detected from single-DNA-tethered nanodumbbells. A well-defined gold nanobridged nanogap particle has also been used to generate the highly stable and reproducible SERS signal with enhancement factors greater than 1.0×10^8 , which is sufficient for single-molecule detection.⁵⁵

SWNTs have several advantages when used as optical sensors, such as photo-stable near-infrared emission for prolonged detection through biological media. Molecular adsorption can be transduced into an optical signal by perturbing the electronic structure of the nanotubes. A single-molecule detection of H_2O_2 has been achieved by stepwise near-infrared photoluminescence quenching of surface-tethered DNA–SWNT complexes. These measurements demonstrate single-molecule detection of H_2O_2 in real time within live 3T3 cells and provide promise for new classes of biosensors with the single-molecule level of sensitivity.²⁰

Carbon nanotube field-effect transistor is an alternative way for the detection of DNA hybridization at the single-molecule level. Shepard and co-workers¹⁹ presented a single-molecule label-free bioanalytical system with a carbon nanotube

field-effect transistor. By covalently attaching a single stranded probe DNA sequence to a point defect in a carbon nanotube, two-level fluctuations in the conductance of the nanotube could be measured in the presence of a complementary DNA target. This technique was label-free and could be used to probe single-molecule dynamics at microsecond timescales.

5. Applications of biofunctional nanomaterials in biosensing

5.1 DNA/miRNA assay

The specific sequence detection of DNA has attracted considerable interest due to its broad applications in molecular diagnostics, genetics therapy, and early screening of cancers. It is highly desirable to develop an ultrasensitive detection method for specific DNA sequences, especially for low-abundant DNA. Some signal amplification strategies have been designed for the detection of DNA at low levels. For example, several enzyme-based sensors have been developed for the detection of femtomolar DNA targets.^{9,18} In order to improve the specificity and sensitivity of DNA detection, a molecular beacon, a single-stranded oligonucleotide hybridization probe with a stem-loop structure, has been introduced for the design of a DNA sensing strategy by combining with some signal amplification strategies.¹⁷

CNTs can be used as carriers for both numerous enzyme tags and the accumulation of enzymatic reaction product. The support and preconcentration functions of CNTs reflect their large specific surface area and have been illustrated using the ALP enzyme tracer. The favorable response of DNA target indicates a remarkably low detection limit of around 1 fg mL^{-1} (54 aM), *i.e.*, 820 copies or 1.3 zmol in the $25 \mu\text{L}$ sample.¹⁸ Based on the specific recognitions of target DNA and streptavidin to a biotin labeled molecular beacon and signal amplification of

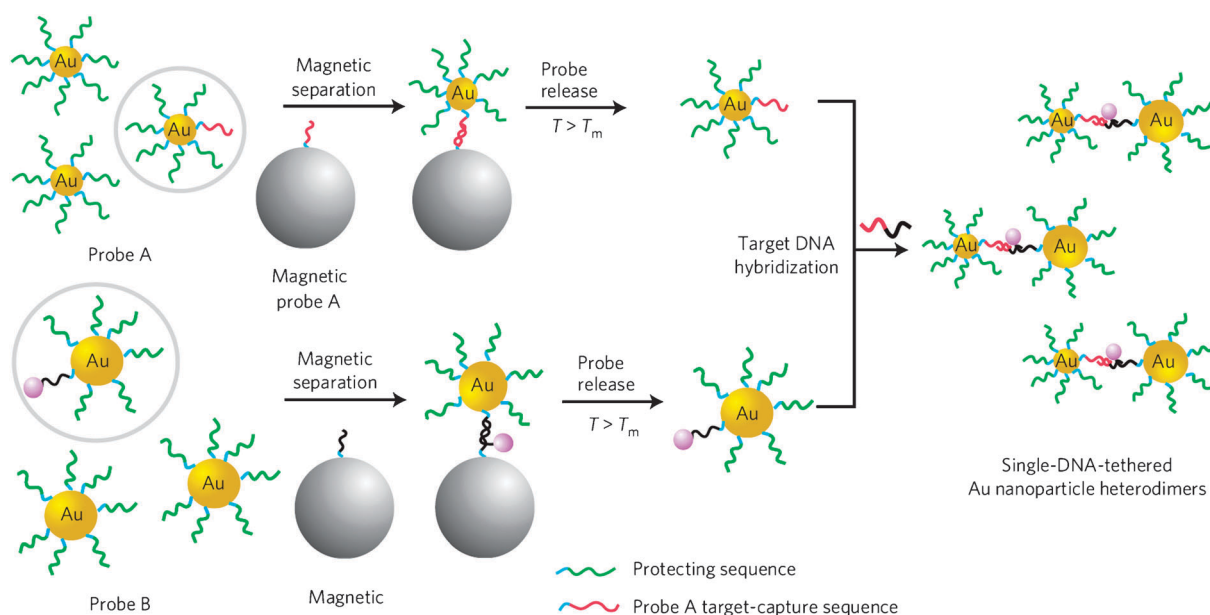


Fig. 5 The high-yield synthetic scheme for the Au nanoparticle heterodimers using stoichiometric DNA modification and magnetic purification for SERS-based single-molecule detection and bioassays [reprinted with permission from ref. 54].

(rather than diffusion-controlled) interaction between the substrate and the enzyme. Under higher rotation speeds, the sensitivity of the antibody detection can be further improved.

SPR utilizing nanoparticle–antibody labels for signal amplification in immunoassays is an emerging approach for detecting proteins in biomedical samples. A simple method has been proposed by using superparamagnetic particles, a commercial SPR flow sensor, and off-line analyte capture to attain ultrahigh sensitivity and an ultralow detection limit for a cancer biomarker protein in serum. The ultralow detection limit of 10 fg mL^{-1} (*ca.* 300 am) is related to off-line reduction of non-specific binding combined with clustering of the supermagnetic particles on the SPR chip.⁴

Surface plasmon (SP) induced ECL enhancement has been applied for ultrasensitive detection of thrombin. The system shows 5-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 nanoparticles. The decrease of ECL signal is logarithmically linear with the concentration of thrombin in a wide range from 100 aM to 100 fM.⁶⁰ To utilize the advantages of both semiconductor nanomaterials and MNPs, a hybrid nanostructure of $\text{Fe}_3\text{O}_4/\text{CdSe}-\text{CdS}$ was employed to fabricate an ECL immunosensor for CEA detection with high selectivity, excellent stability, and good reproducibility.⁶¹

Since reliable disease diagnosis often requires the identification of the levels of a number of molecular markers, the development of high-throughput techniques for multiplexing analysis has become another key direction of research in biosensors. A series of disposable immunosensor arrays have been designed for ultrasensitive multiplexed measurements of tumor markers by using streptavidin-functionalized silver-nanoparticle-enriched carbon nanotubes and enzyme–AuNP catalyzed deposition of silver nanoparticles as trace tags.^{30,48} At the single bead level based platform, multiplexed sandwich immunoassays can be realized by using ECL as a readout mechanism. This approach should enable the analysis of dozens of analytes simultaneously.⁶²

5.3 Cytosensing and cellular carbohydrate expression

In cytosensing, functional nanomaterials are mainly used in two roles as nanoscaffolds for immobilization of cells and as nano-probes to combine the specific recognition, signal transduction and signal amplification abilities. On the basis of the dual signal amplification of SWNTs and enzymatic catalysis, an electrochemical cytosensing strategy is designed based on the specific recognition of integrin receptors on a cell surface by arginine-glycine-aspartic acid-serine (RGDS)-functionalized SWNTs. The cytosensor can respond down to $620 \text{ cells mL}^{-1}$ of BGC-823 human gastric carcinoma cells with a linear calibration range from 1.0×10^3 to $1.0 \times 10^7 \text{ cells mL}^{-1}$.²¹ Further, four HRP lectins (concanavalin A (ConA), dolichos biflorus agglutinin (DBA), peanut agglutinin (PNA), and wheat germ agglutinin (WGA)) can be used for simultaneous multiple analysis at screen-printed carbon electrodes (SPCEs) (Fig. 7a).⁶³ The designed electrochemical cytosensor array has been used for analyzing the dynamic change of the K562 cell-surface glycome during erythroid differentiation induced by sodium butyrate (NaBut). As shown in Fig. 7b, upon treatment the

DPV signals for HRP PNA and HRP DBA progressively increased and tended to steady magnitudes after three days, while the DPV signals for WGA- and ConA-recognizing glycans showed small changes. Thus the expression of PNA- and DBA-recognizing glycans (O glycans) on erythroid-differentiated K562 cell surfaces significantly increased, while WGA- and ConA-recognizing glycans were expressed at relatively steady levels. This facile cytosensor array has the potential to become a powerful and pragmatic tool to decode the cell surface glycome and discover potential glycan biomarkers and novel therapeutic targets.

Another strategy for *in situ* analysis of cell surface carbohydrate has been developed by integrating a biomimetic carbohydrate monolayer, competitive recognition in a one molecule-two surfaces format.¹⁵ The proposed method exhibits a sensitive response to K562 cell surface carbohydrates with a wide linear range down to $102 \text{ cells mL}^{-1}$. The average Con A binding capacity of a single K562 cell can be estimated to correspond to 6.9 pg or 2.3×10^{10} mannose moieties, which is slightly higher than $(4.9 \pm 0.6) \times 10^9$ mannose moieties obtained with an enzymatic method, due to the inevitable loss of mannose in the destructive sample preparation for enzymatic analysis.

Since photoacoustic imaging has higher spatial resolution in deep tissues (up to 3 cm) than other optical modalities, a new platform using targeted MNPs in combination with two-colour photo-acoustic flow cytometry has been developed for *in vivo* magnetic enrichment and detection of rare circulating tumour cells from a large pool of blood. The approach has potential for the early diagnosis of cancer and the prevention of metastasis in human.⁶⁴

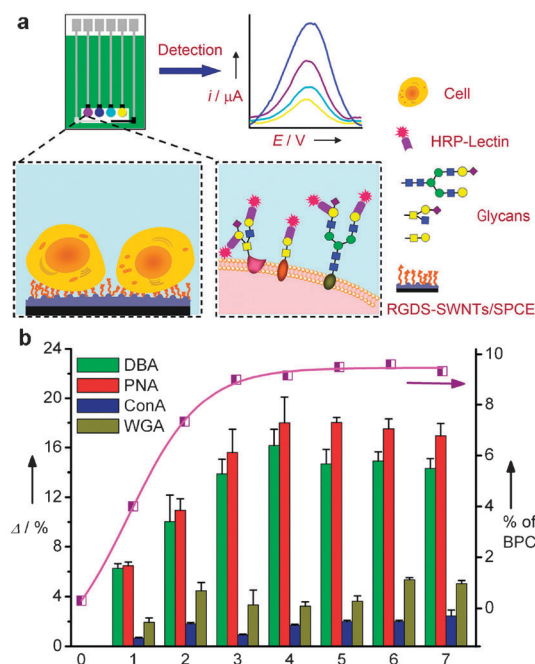


Fig. 7 (a) Schematic representation of the electrochemical cytosensor array for cell-surface glycan analysis, and close-up illustrations of cells captured on RGDS-SWNTs/SPCE and HRP-lectin binding with cell-surface glycans. (b) Glycan expression changes on K562 cells treated with NaBut from 1 to 7 days monitored with the designed arrays, and percentage of benzidine-positive cells (BPC) during NaBut treatment [reprinted with permission from ref. 63].

6. Conclusions and perspectives

The need for ultrasensitive bioassays and the trend towards miniaturized assays has made the biofunctionalization of nanomaterials one of the hottest fields of research. A wide variety of nanoscaled materials with different sizes, shapes and compositions have been introduced into biosensing for signal amplification. Two approaches including noncovalent interaction and covalent interaction can introduce functional groups to the surface of NPs. The biofunctional NPs can be used as carriers or tracers, catalysts, electronic conductors and optical emitters, and produce a synergic effect among catalytic activity, conductivity and biocompatibility to result in significant signal amplification. These properties endow nanomaterials with excellent electronic, optical, and photoelectrochemical signal transduction abilities for design of a new generation of biosensing devices and application in the detection of DNA, protein, cells and other important biomolecules.

There are many challenges still in the development of ultrasensitive bioassays based on biofunctional nanomaterials. (i) Many proteins or other complex biological systems require a physiological environment and a minimum degree of biocompatibility of the solid substrate. Therefore, in order to increase the biocompatibility, a significant direction to explore is a mild biofunctional way to fix the biomolecules on the surface of nanomaterials. The as-prepared conjugation must fulfill the criteria of efficiency and selectivity, stability of the resulting bond, and biocompatibility. (ii) It is desirable to seek a novel nanomaterial with sufficient binding sites for functionalization, especially for the low-toxicity, eco-friendly alternatives such as silicon and carbon nanomaterials. (iii) Since the simultaneous analysis is required in practice, it is of key interest for the development of high-throughput techniques for the parallel analysis of numerous components in samples. The possibility to control and tune these unique optical and electronic properties of metal or semiconductor NPs through their dimensions paves the way to the application of NPs as versatile analytical probes. (iv) Due to the diverse properties of different nanomaterials, utilizing two or more types of nanomaterials can enhance the good qualities as well as offset the insufficiency of each individual nanomaterial, which can produce better results than that using only one type of nanomaterial. (v) Mimicking biological signal transduction, nanomaterials-based autocatalytic systems, in which each step produces a product that acts not just as a template or just as a stoichiometric trigger, but rather is a catalyst (or activates a catalyst) to produce more products, provide another opportunity for exponential signal amplification. With the demand in life sciences and clinical diagnosis, the ultimate goal of this field is the utilization of nanomaterials which not only enhances the biosensing capabilities compared with conventional platforms, but also brings out new approaches such as miniaturization, reagent-less biosensing and single-molecule detection.

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