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REVIEW

Nanotechnology-Enhanced No-Wash Biosensors for *in Vitro* Diagnostics of Cancer

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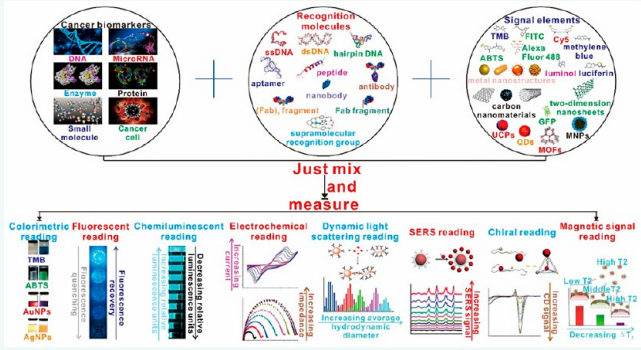
ABSTRACT: ***In vitro*** **biosensors have been an integral component for early diagnosis of cancer in the clinic. Among them, no-wash biosensors, which only depend on the simple mixing of the signal generating probes and the sample solution without additional washing and separation steps, have been found to be particularly attractive. The outstanding advantages of facile, convenient, and rapid response of no-wash biosensors are especially suitable for point-of-care testing (POCT). One fast-growing field of no-wash biosensor design involves the usage of nanomaterials as signal amplification carriers or direct signal generating elements. The analytical capacity of no-wash biosensors with respect to sensitivity or limit of detection, specificity, stability, and multiplexing detection capacity is largely improved because of their large surface area, excellent optical, electrical, catalytic, and magnetic properties. This review provides a comprehensive overview of various nanomaterial-enhanced no-wash biosensing technologies and focuses on the analysis of the underlying mechanism of these technologies applied for the early detection of cancer biomarkers ranging from small molecules to proteins, and even whole cancerous cells. Representative examples are selected to demonstrate the proof-of-concept with promising applications for *in vitro* diagnostics of cancer. Finally, a brief discussion of common unresolved issues and a perspective outlook on the field are provided.**



KEYWORDS: *nanotechnology, nanomaterial, signal transducer, biosensor, no-wash detection, point-of-care testing, cancer biomarker, in vitro diagnostics*

Cancer has been one of the leading causes of death around the world.[1] Early and accurate diagnosis can augment the chances of successful treatment, thereby increasing overall cancer survival rates and decreasing patient suffering. Existing clinical cancer diagnostic methods mainly employ clinical imaging techniques and morphological analysis of the cell or tissue (cytology or histopathology).[2,3] However, these methods suffer from relatively low sensitivity, which tend to cause misdiagnosis and thus are not appropriate for early detection of cancer.[4]

Biosensors have been considered a major thrust of clinical early diagnostics. *In vitro* biosensors utilize samples of blood, urine, or tumor tissues, containing proteins, DNAs/RNAs, enzymes, small molecules, as well as cells, to serve as specific biomarkers for cancer detection, monitoring, and prognosis.[5−7] Heterogeneous biosensors, the most commonly used type of *in vitro* biosensor, rely on the diffusion of target molecules in sample solution toward a solid surface for signal generation, where the detection signal can be separated from the background signal through a wash step. A typical example of a heterogeneous biosensor test is the enzyme-linked immunosorbent assay (ELISA), which has been one of the most widely used assay formats in clinical biomarker detection. However, the major challenge of heterogeneous biosensors is the requirement of repeated separation and wash steps, which makes the analytical procedure rather time-consuming and labor-intensive and is inherently more prone to error that may lead to cancer misdiagnosis.[8,9]

No-wash biosensor assays are performed through simple mixing of the signal generating probes and the sample solution, and the production of detectable signal occurs in solution rather than on a solid surface. Compared with conventional heterogeneous biosensors, the biggest advantages of no-wash biosensors are that they are simpler, more convenient, and faster, making them more suitable for point-of-care testing (POCT). The detectable signals are produced following the binding interaction of target analytes and detection probes, which avoid the need for a wash step to separate the

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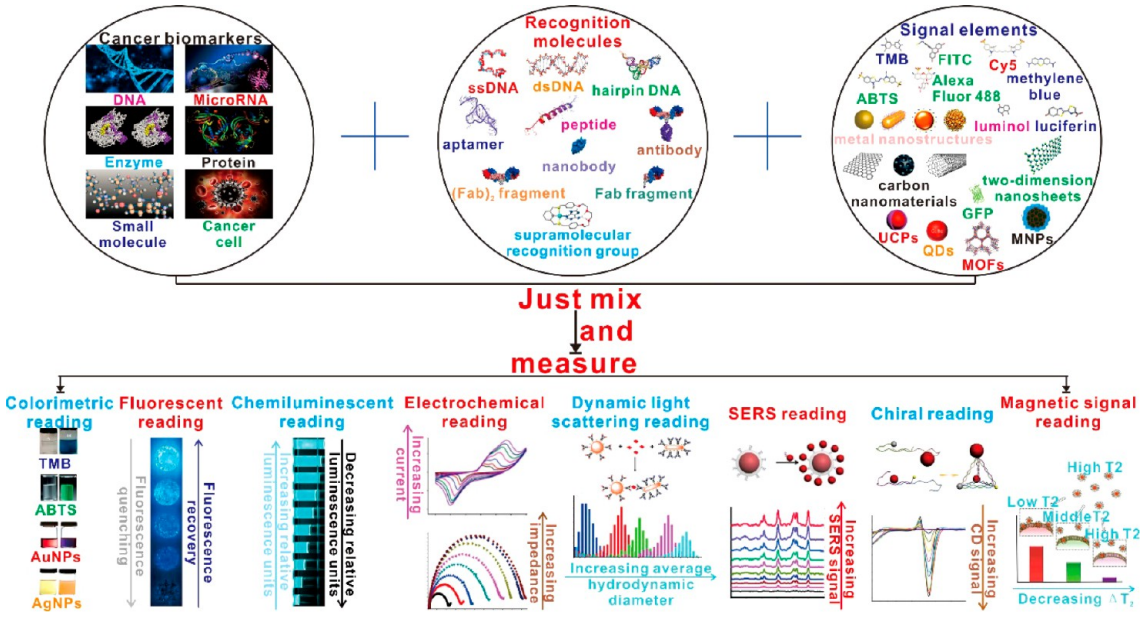

Figure 1. Schematic illustration of various no-wash biosensors for cancer biomarkers *in vitro*.

background signal. Moreover, the binding interactions of target analytes and detection probes occur in solution, resulting in more rapid binding kinetics owing to the three-dimensional (3D) diffusion of target molecules and signaling probes, further shortening total assay times. The past few decades have witnessed significant and tremendous progress in this field. Various promising no-wash biosensors have been reported for the *in vitro* detection of cancer biomarkers ranging from small molecules to proteins, and even cancer cells. Correspondingly, with the advent of nanotechnology, enormous breakthroughs in the design of no-wash biosensors have been attained through the exploration of different types of nanomaterials such as noble metal nanoparticles, quantum dots (QDs), upconversion phosphors (UCPs), carbon nanomaterials, graphene-like two-dimensional (2D) layered nanomaterials, metal−organic frameworks (MOFs), as well as magnetic nanoparticles (MNPs). The analytical performances of no-wash biosensors including sensitivity, specificity, and multiplexing capacity are largely improved owing to their distinct physical and chemical properties and excellent optical, electrical, catalytic, and magnetic properties. Nanomaterials and nanotechnology endow exciting opportunities to explore advanced no-wash biosensor strategies through nanomaterials as signal amplification carriers or simple signal generating elements.

Nanomaterials and nanotechnology have facilitated major advancements in the field of biosensors, and several reviews have been presented elsewhere.[3,10−14] However, to the best of our knowledge, a critical, systematic, and comprehensive review focusing on the exciting progress in the field of nanotechnology-enhanced no-wash biosensors for *in vitro* diagnosis has not been previously completed. Thus, in this review, we will focus on some major nanomaterial-based no-wash biosensor technologies for cancer biomarker detection. Although the synthesis, properties, and surface modification of nanomaterials are vital for the development of biosensors, in this review we aim to emphasize the detection strategies of various no-wash biosensors, including colorimetric, fluorescent, chemiluminescent, electrochemical, magnetic, light scattering, and chirality-based biosensors (Figure 1). The emphasis of this review is placed on the discussion of the integration mechanism of various signal transduction approaches in no-wash biosensors, especially with respect to the role of nanomaterials. Some representative examples are selected to demonstrate the proof-of-principle with promising applications for early detection of cancer biomarkers *in vitro*. We hope this comprehensive review can help researchers become acclimated with the current status of the field and help potential users find the available and appropriate analytical tools to meet their application requirements.

COLORIMETRIC NO-WASH BIOSENSORS

Colorimetric biosensors are based on target-induced color intensity change, which can be easily distinguished by the naked eye for qualitative assay or by simple spectrometry for quantitative analysis,[15] making the colorimetric biosensor the most important assay platform for field analysis or rapid screening of tumor biomarkers. No-wash colorimetric biosensors have been drawing increasing attention for *in vitro* detection of cancer biomarkers, as they are simpler, cheaper, and easier to automate in comparison with heterogeneous colorimetric biosensors.[16,17] Conventional no-wash colorimetric biosensors depend on enzyme-catalyzed organic chromogenic substrates such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 3,3′,5,5′-tetramethylbenzidine sulfate (TMB) to form colored products, which suffer from relatively low detection sensitivity.[18,19]

Noble metal nanoparticles, including gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs), are a class of excellent signal indicators for colorimetric biosensors because they exhibit high extinction coefficients and special localized surface plasmon resonance (LSPR).[20,21] An obvious color change of noble metal nanoparticles is easily achieved by altering the nanoparticle size, morphology, interparticle distance, as well as the local dielectric environment.[22] When in close proximity, there exists strong interparticle plasmon coupling and an associated perturbation in the LSPR band of the ensemble, leading to a red-shift in the absorbance peak.[23] Capitalizing on this feature, many noble metal nanoparticle-

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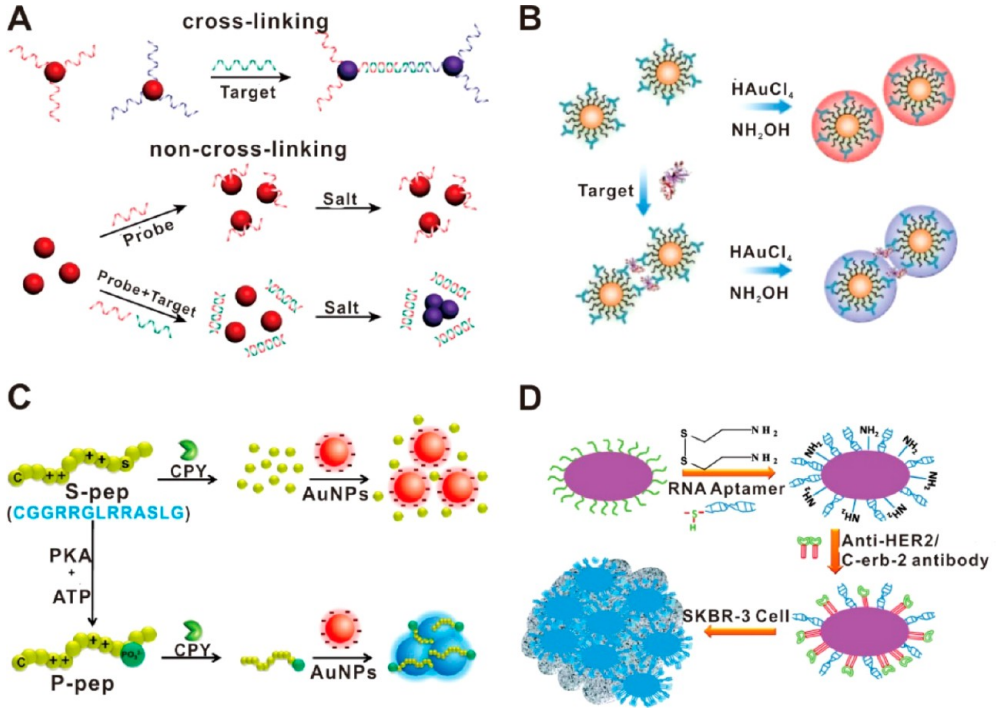

Figure 2. (A) Colorimetric biosensor for DNA based on target-induced crossing or non-crossing aggregation of AuNPs. Reprinted with permission from ref 34. Copyright 2012 American Chemical Society. (B) No-wash colorimetric immunosensor for CEA based on the controllable growth of AuNPs. Reprinted with permission from ref 72. Copyright 2016 Ivyspring International Publisher. (C) Colorimetric biosensor for monitoring PKA activity based on carboxypeptidase Y (CPY) and unmodified AuNPs. Reprinted with permission from ref 74. Copyright 2013 Elsevier B.V. (D) Colorimetric sensing of SK-BR-3 breast cancer cell line with multifunctional oval-shaped AuNP. Reprinted with permission from ref 84. Copyright 2010 American Chemical Society.

based no-wash colorimetric sensors have been developed for determining proteins, enzymes, nucleic acids, small molecules, pathogens, as well as cells.[24]

TARGET-INDUCED NANOPARTICLE AGGREGATION

AuNPs. AuNPs have been widely used as colorimetric probes for the detection of a variety of analytes owing to their facile synthesis, easy surface modification, good aqueous solubility, and excellent biocompatibility.[25−29] Specifically, AuNPs exhibit an ultrahigh extinction coefficient (*ca.* 10^8 cm^{-1} M^{-1}), which is 3 orders of magnitude higher than those of traditional organic dyes (*ca.* 10^5 cm^{-1} M^{-1}).[30] This excellent property makes AuNP-based colorimetric biosensors ideal for naked-eye visibility and further provides high sensitivity at the nanomolar level.[31−33] Target-induced AuNP aggregation can result in a dramatic LSPR red shift with a visible color change from red to blue, enabling a simple colorimetric detection platform.[34,35] This signal readout can be conducted by UV−vis spectroscopy or by the naked eye. A significant contribution to AuNP aggregation-based colorimetric sensors was that of Mirkin and co-workers,[36−38] who first demonstrated and applied this strategy for colorimetric detection of target DNA. In this example, the presence of target DNA molecules can induce AuNPs modified with complementary single-stranded DNA (ssDNA−AuNPs) to cross-link and form AuNP aggregates (Figure 2A). Inspired by this study, many research groups have exploited the same technology to detect the presence of target nucleic acids,[39,40] organic small molecules,[41,42] and to discriminate single or multiple point mutations.[43,44] Non-cross-linking-based AuNP aggregation is another way to construct no-wash colorimetric biosensors, which depends on target-induced AuNP stabilization loss. Maeda and co-workers[45] found that ssDNA can adsorb onto the AuNP surface *via* DNA base−Au interactions to stabilize AuNPs, whereas double-stranded DNA-modified AuNPs (dsDNA−AuNPs) spontaneously aggregate in a non-cross-linking manner after the addition of a high-concentration NaCl (Figure 2A). Based on this finding, a series of AuNP aggregation-based colorimetric detection platforms have been employed to measure nucleic acids[46] and small molecules.[47] Recently, an interesting study compared the two AuNP aggregation modes using DNA hybridization and found that the cross-linking aggregation mode occurred more rapidly than the non-cross-linking aggregation mode in the presence of a small number of cross-linkers or complementary DNAs. On the contrary, the non-cross-linking aggregation exhibited more rapid color change than the cross-linking counterpart with a large number of DNAs.[48] This finding will help researchers choose the appropriate aggregation mode to apply ssDNA−AuNPs for colorimetric biosensors under given conditions.

The simple cross-linking or non-cross-linking AuNP aggregation provides a rapid and convenient no-wash colorimetric assay platform. However, the detection sensitivity is limited to the nanomolar or subnanomolar level. In most cases, DNA biomarkers direct from clinical samples are undetectable by these methods because their concentrations are found in attomolar to picomolar levels. To increase the analytical sensitivity, various signal amplification strategies have been introduced including enzyme-based signal amplification (*e.g.*, exonuclease III,[49] nicking endonucleases,[39,50] ligases,[51−53] and polymerases[54,55]) and enzyme-free signal amplification (HCR[56,57] and CHA[58,59]) methods. With the aid of different

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signal amplification strategies, the detection sensitivity of AuNP aggregation-based colorimetric biosensors has reached the required levels of sensitivity, ranging from picomolar to attomolar and even zeptomolar detection capabilities.

MicroRNAs (miRNAs) are a class of small non-protein coding RNAs with a length of 17−25 nucleotides, which have been largely regarded as biomarkers for cancer diagnosis and prognosis since miRNA is associated with cancer initiation, tumor stage, as well as tumor response to treatments.[60] Compared with DNA detection, miRNA detection is very difficult due to their intrinsic small size, sequence homology, and low relative concentration in total RNA samples.[61] Similar to DNA detection, AuNP aggregation-based colorimetric sensors provide an excellent option for the detection of trace amounts of miRNA by combining with enzyme-mediated signal amplification platforms including polymerase and nicking endonucleases,[62] as well as duplex-specific nucleases (DSN).[63,64]

Protein biomarker detection is of great significance in clinical cancer diagnosis. Abnormal expression of proteins may be associated with cancer. For example, the overexpression of alpha-fetoprotein (AFP) is an important signal for the diagnosis of primary liver cancer. Currently, immunoassay is the most prevalent clinical tool for protein detection, including systems such as ELISA. However, the moderate sensitivity of conventional ELISA restricts its applications for the detection of low concentrations of protein biomarkers indicative of early stage cancer. Recently, AuNP-based plasmonic ELISAs exhibiting ultrahigh sensitivity were developed, largely improving the sensitivity of conventional ELISA for target biomarker detection.[65,66] However, these assays are also based on heterogeneous formats like traditional ELISA, which rely on time-consuming incubation and washing steps. Compared with small molecules or DNA, both target protein and corresponding antibodies possess larger molecular dimensions, thus the interparticle distance is larger than the particle size after the formation of antibody−protein−AuNP complex, thereby giving rise to the disappearance of strong LSPR phenomenon from interparticle plasmon coupling. Thus, it is not feasible to directly induce antibody-modified AuNP aggregation for colorimetric detection by using target proteins. In order to use AuNP cross-linking aggregation-based no-wash colorimetric biosensors for protein detection, several interesting and smart designs have been described to decrease the interparticle spacing of protein cross-linking AuNP complexes, thus generating greater interparticle plasmon coupling. Methods include using synthetic smaller affinity biorecognition elements (*e.g.*, aptamer and peptide) to replace large-sized antibodies,[67−69] applying high concentrations of NaCl to destroy the hydration layer of the AuNP surface,[70] and the selection of large-sized AuNPs.[71] Recently, Liu *et al.*[72] reduced the interparticle distance of cross-linked AuNPs by controlling the growth of AuNPs on cross-linked AuNPs. The enlargement of AuNP size from the growth in solution decreased interparticle spacing of the cross-linked AuNPs, thereby producing a strong interparticle plasmon coupling for colorimetric detection of cancer biomarker, carcinoembryonic antigen (CEA) (Figure 2B). Enzymes are a particular type of protein with catalytic activity. The anomalous change in enzyme activity is correlated with uncontrolled cell division, angiogenesis, and metastasis that gives rise to the growth of cancer cells. Thus, it is very important to develop highly sensitive detection methods for early and rapid monitoring of enzyme activity. Telomerase is a reverse transcriptase that can catalyze the telomeric repetitive sequence TTAGGG. The overexpressed telomerase is correlated with most known human cancers (>85%), which makes it a valuable biomarker for cancer diagnosis. AuNP aggregation-based colorimetric biosensors have been explored to detect telomerase activity because of their superiority over instrument-based analysis, including enhanced simplicity, sensitivity, and low cost. For example, Zhang *et al.*[73] reported a no-wash colorimetric sensor for telomerase activity assay through exonuclease I-mediated telomerase primer-modified AuNP aggregation. So far, many efforts have been devoted to similar no-wash AuNP aggregation-based colorimetric biosensors for other cancer related enzymes, such as protein kinase,[74−77] caspase-3,[78] glycosidases,[79] matrix metalloproteinase (MMP)-7,[80] cathepsin B,[81] and gelatinase.[82] Zhou *et al.* developed a colorimetric biosensor for monitoring protein kinase activity through phosphorylation-induced suppression of carboxypeptidase Y (CPY) cleavage to stimulate AuNP aggregation with a visible color change from red to blue (Figure 2C).[74]

A method for the early and accurate detection of cancerous cells would revolutionize the clinical diagnosis and treatment of cancer. The detection of whole cancer cells under the assistance of AuNP aggregation-based colorimetric biosensor systems provides a potential horizon in the field of diagnosis. Compared with other targets such as nucleic acids or proteins, tumor cells exhibit larger surface area and more binding sites, thereby serving as a carrier for AuNP self-assembly through biorecognition molecules (*e.g.*, antibody or aptamer) that cause a LSPR shift due to interparticle plasmon coupling. Medley *et al.*[83] reported a direct measurement of cancer cells using a AuNP aggregation-based colorimetric sensor. However, the aptamers used in this case demonstrated only weak binding affinity for cancerous cells and thus led to low signal, which was insufficient for early and highly sensitive detection of CCRF-CEM cells. To address this issue, Lu *et al.*[84] proposed to use monoclonal anti-HER2/c-erb-2 antibody and S6 RNA aptamer-conjugated multifunctional oval-shaped AuNPs to achieve multivalent binding between AuNPs and target cells for highly sensitive detection of SK-BR-3 breast cancer cells (Figure 2D). Several other AuNP-based colorimetric strategies have also been reported for early and sensitive detection of cancerous cells *in vitro*.[85,86]

AgNPs. From an optical sensing point of view, AgNPs are also good candidates for colorimetric detection in addition to AuNPs since AgNPs exhibit much higher extinction coefficients relative to AuNPs of the same size.[87] Likewise, AgNPs also show an interparticle distance-dependent color change. Dispersive AgNPs have LSPR absorption peaks at around 400 nm, corresponding to a yellow color. However, AgNP aggregation induced by target analytes will result in a rapid color change from yellow to orange. Based on this principle, AgNP aggregation-based colorimetric biosensors have been used for the detection of a variety of biomolecules, such as DNA/RNA,[88−90] enzyme,[91−93] and small molecules.[94,95] For example, Miao *et al.* described a AgNP aggregation-based colorimetric biosensor for sensitive miRNA analysis with the help of HCR technology.[90] Efforts were also made to employ AgNP aggregation strategy for simple, sensitive colorimetric assay of cancer-related enzymes.[92,93] Nevertheless, compared with AuNPs, much less attention has been paid to AgNP-based colorimetric biosensors owing to the poor stability of AgNPs,

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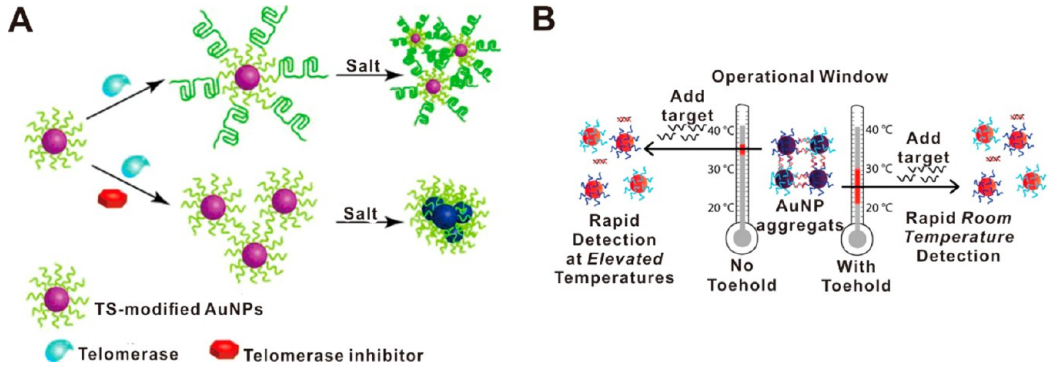

Figure 3. (A) Colorimetric biosensor of telomerase activity based on TS primer-modified AuNPs. Reprinted with permission from ref 103. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA. (B) Colorimetric biosensor for DNA detection through target-induced disassembly of DNA-AuNPs. Reprinted with permission from ref 40. Copyright 2015 American Chemical Society.

oxidation and chemical degradation during functionalization, and their broad size distribution following synthesis.

TARGET-INDUCED NANOPARTICLE DISPERSION

AuNPs. Compared with AuNP aggregation-based biosensing, target-induced AuNP dispersion strategies may produce higher sensitivity owing to the lower possibility of type I error. One simple method for AuNP dispersion-based colorimetric sensing is the use of target analytes that inhibit AuNP aggregation. In the presence of target molecules, the color of solution is red; in contrast, without targets, a blue solution is observed. Enzyme-mediated inhibition of AuNP aggregation is an effective method for AuNP dispersion-based colorimetric biosensing. Guarise and colleagues[96] first demonstrated a simple enzyme-inhibited AuNP aggregation for colorimetric protease assay by cleaving the peptide substrates modified on the AuNP surface. In similar fashion, many attempts have been made to analyze various cancer-related enzymatic activities, including methyltransferase,[97] protein kinase C,[98] adenosine deaminase,[99] alkaline phosphatase (AP),[100] MMP,[101] peptidases,[102] telomerase,[103−105] as well as hyaluronidase.[106] For instance, Wang *et al.*[103] described a no-wash colorimetric method for visual analysis of human telomerase activity using primer-modified AuNPs, where the presence of target could lead to the elongation of primer preconjugated on the surface of AuNPs that can fold into a G-quadruplex to inhibit AuNP aggregation (Figure 3A). Target protein-triggered AuNP dispersion was also used to develop AuNP-based colorimetric sensors. Chang *et al.*[107] fabricated a AuNP dispersion-based colorimetric biosensor for human chorionic gonadotropin (HCG) by employing a competitive combination of target and citrate-capped AuNPs with a peptide probe that can cause AuNP aggregation. Wei *et al.*[108] described a simple colorimetric strategy for the detection of vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) based on the combination of target and peptide-decorated AuNPs to inhibit the cucurbit[8]-uril-mediated AuNP aggregation. Moreover, Zhang and co-workers[109] designed a sensitive colorimetric aptasensor for direct visual analysis of CCRF-CEM cancer cells by using target-inhibited AuNP aggregation in combination with cell-triggered cyclic enzymatic signal amplification.

Another method for AuNP dispersion-based colorimetric biosensing is target analyte-induced disassembly of AuNP cross-linkers or aggregates to produce an obvious blue to red color shift. To achieve disassembly of DNA-AuNP aggregates, various means have been thoroughly investigated, including temperature,[110] DNA displacement reaction,[111] enzymes,[112] and target-induced aptamer structure switching.[113] For example, Jin *et al.*[110] constructed a highly sensitive and selective DNA detection method by increasing the incubation temperature above the melting dissociation temperature of the hybridized DNA. Hazarika and co-workers[111] first used a DNA displacement reaction to realize the reversible switching of DNA−AuNP aggregates, and Zhou *et al.*[114] improved upon this approach for colorimetric detection of DNA. Recently, Lam *et al.*[40] also reported simple visualization of low pM concentrations of target DNA with strand displacement-triggered disassembly (Figure 3B). Enzyme-triggered dispersion is another direct and effective way to disassemble AuNP aggregates. Taking advantage of this strategy, Laromaine *et al.*[112] and Mirkin *et al.*[115] successfully monitored protease and endonuclease activities, respectively.

OUTLOOK OF COLORIMETRIC NO-WASH BIOSENSORS

Colorimetric no-wash biosensors provide great potential for various diversified applications, such as detecting DNA, enzyme activity, protein, and cells, because these methods are low-cost, fast, and simple. However, these no-wash colorimetric biosensors suffer from great challenges that need to be addressed before their widespread clinical implementation. First, human body fluid samples often present a background signal. When these samples are directly used for no-wash colorimetric detection, these background colors may disturb the detection signal color, thereby biasing detection results. Some effective methods such as using diluted samples can be employed to eliminate interference. Second, the stability of nanoparticles in sample solution is vital to no-wash assays. Nanoparticles tend to aggregate nonspecifically in complex biological samples that result in inaccurate readouts. Thus, to obtain a higher quality of nanoparticle with high stability and strong anti-interference capability, more effective surface modifications and conjugation strategies should be explored. Third, biorecognition molecules modified onto nanoparticle surfaces may be displaced by biothiols present in patient samples, thereby reducing the recognition capacity. Fourth, for AuNP or AgNP aggregation-mediated colorimetric biosensors, the aggregation process is dynamic and results in the formation of unstable large-sized aggregates that tend to precipitate out of aqueous solutions. Thus, the color change of the solution is time dependent, which seriously confounds the accuracy and reliability of quantitative detection of target analytes. Fifth, for

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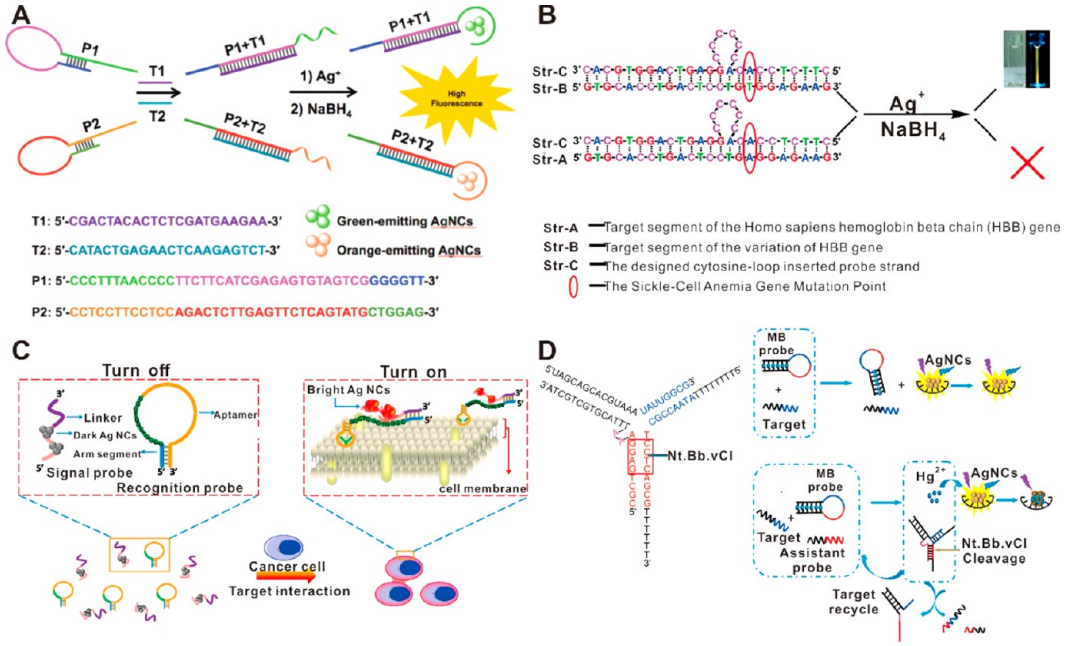

Figure 4. (A) Fluorescent AgNC-based MBs for multiplexed analysis of DNA. Reprinted with permission from ref 130. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA. (B) DNA-templated AgNC *in situ* synthesis-based fluorescence biosensor for the sickle cell anemia gene mutation. Reprinted with permission from ref 131. Copyright 2010 American Chemical Society. (C) Hybridization-enhanced DNA−AgNC fluorescence for cancer cell detection. Reprinted with permission from ref 151. Copyright 2013 American Chemical Society. (D) Target-induced fluorescence quenching-based biosensor for miRNA. Reprinted with permission from ref 155. Copyright 2013 Elsevier B.V.

AuNP dispersion-mediated colorimetric biosensors, the disassembly of AuNP cross-linkers or aggregates is quite slow, which requires a long time to complete dissociation. Finally, mass-scale synthesis of stable functionalized nanoparticles is necessary for their wide use in colorimetric biosensors. Although noble metal nanoparticle-based colorimetric biosensors have achieved great success in cancer biomarker detection, designing nanoparticles with strong colorimetric distinguish ability will help explore promising frontiers in no-wash colorimetric sensors.

FLUORESCENT NO-WASH BIOSENSORS

Compared with absorbance-based biosensors, fluorescence biosensors are more sensitive (up to 1000 times higher than absorptiometry) and can even reach single-molecule detection level. Fluorescence biosensors have been a well-established and prevailing optical technology that play a key role in biomedical diagnostics. Conventional fluorescent assays employ enzymes to catalyze fluorogenic substrates to generate detectable fluorescent molecules as signal output. However, conventional organic fluorogenic substrates suffer from relatively low luminescence intensity and are vulnerable to photobleaching. Moreover, these assays are based on heterogeneous formats that often are subjected to false-positive signals arising from nonspecific binding and multiple washing steps. To overcome these problems, fluorescent no-wash biosensors are proposed as effective options to detect target molecules directly and without any washing procedures. Up to now, many efforts have been devoted to exploring simple and rapid no-wash fluorescent biosensors. Particularly, with the rapid development of nanoscience and nanotechnology, a wide range of fluorescence no-wash biosensors have emerged with excellent sensitivity, specificity, and simple operation for *in vitro* detection of cancerous biomarkers.

NANOPARTICLES AS FLUOROPHORES

Fluorescent metal nanoclusters (MNCs) are an emerging class of luminescent nanomaterials that are composed of several to a few hundred atoms.[116] In recent years, MNCs have drawn increasing attention due to their special physical and chemical properties, optical and electronical properties, and molecule-like characteristics.[117,118] These excellent properties make them ideal fluorescent nanomaterials for promising applications in biological analysis and biological imaging.

Gold nanoclusters (AuNCs) have attracted great interest as a generation of fluorescent probes for biolabeling and biosensing because of their ultrasmall size, bright photoluminescence, good biocompatibility, and photostability.[119] West *et al.*[120] reported a no-wash fluorescent sensing method for DNA detection using DNase 1 as a reducing agent and stabilizers to prepare two protein-stabilized AuNCs consisting of 8 or 25 atoms, where the DNase 1:Au_8NCs displayed blue fluorescence, while the DNase 1:Au_{25}NCs showed red fluorescence. Subsequently, Lin and co-workers[121] presented a modified synthetic method for DNA-stabilized AuNCs as ratiometric fluorescent probes for sensitive determination of target DNA. AuNCs were also applied to measure enzymes. Wang *et al.*[122] used protein-protected AuNCs as fluorescent probes for protease detection. In this case, the protein shell could be degraded owing to protease-catalyzed hydrolysis that leads to the fluorescence quenching of AuNCs in the presence of oxygen. Similarly, Wen and colleagues[123] applied peptide-templated AuNCs as a sensitive fluorescent beacon for detection of protein post-translational modification enzymes. Afterward, using a similar method, AuNC-based fluorescence biosensors have also been used to test other tumor-related enzymes, such as phospholipase C,[124] protein kinase,[125,126] caspase 3,[127] as well as casein kinase II.[128]

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Compared to AuNCs, silver nanoclusters (AgNCs) are brighter and can be easily synthesized with different ligands. Currently, AgNC-based fluorescent no-wash biosensors mainly involve the following four signaling methods: nanocluster molecular beacon (MB)-based biosensors, DNA-templated AgNC (DNA−AgNC) *in situ* synthesis-based biosensors, hybridization enhanced fluorescence-based biosensors using guanine-rich DNA sequences, and target-induced fluorescence quenching biosensors. For example, through the phenomenon of photoinduced electron transfer between DNA−AgNCs and G-quadruplex/hemin complexes that leads to a decrease in the fluorescence of AgNCs, Zhang and co-workers[129] developed a nanocluster-based MB for highly sensitive detection of target DNA. After that, Zhang *et al.*[130] designed a MB using DNA−AgNCs for multiple DNA detection, where green-emitting and orange-emitting DNA−AgNCs served as fluorescent hosts, respectively (Figure 4A). Target-induced *in situ* synthesis of AgNCs is another method for the preparation of fluorescent no-wash biosensors. Guo *et al.*[131] designed an interesting DNA probe with an inserted cytosine loop that can be selectively hybridized with target DNA to form duplex DNA scaffolds for site-specific growth of fluorescent AgNCs (Figure 4B). Results found that the generation of fluorescent AgNCs was highly sequence dependent and could selectively recognize a typical single-nucleotide mutation from sickle cell anemia. Afterward, several groups also reported the use of this concept for the analysis of other disease-related DNAs,[132,133] miRNAs,[134,135] enzymes[136,137] and protein biomarkers.[138,139] Ma and co-workers[137] presented an *in situ* synthesized DNA−AgNC-based on−off switch fluorescence biosensor to detect pyrophosphate or AP with the aid of copper. Nevertheless, the detection sensitivity for target-triggered *in situ* synthesis of DNA−AgNCs may be poor because each target DNA generates only one fluorescent AgNC. To increase sensitivity, some signal amplification strategies, such as isothermal exponential amplification[140] and HCR[141] were proposed recently. Another interesting class of AgNC-based fluorescent no-wash biosensor is based on the change in AgNC fluorescence upon DNA hybridization through the use of a G-rich overhang enhancer. Zhang *et al.*[142] demonstrated that fluorescence intensity from DNA−AgNC-based beacons increased by the G-rich sequence through toehold-mediated DNA−AgNCs. In this method, one strand of DNA displaces another in binding to a third strand with partial complementarity to both. Such a sensing system was successfully used for fluorescent detection of target protein with high selectivity and sensitivity. Hereafter, several similar enhanced AgNC-based fluorescent no-wash biosensors were developed for the detection of other clinically significant DNA fragments,[143−145] enzymes,[146−149] proteins,[150] and even tumor cells.[151] Yin *et al.*[151] designed an on−off switch aptamer strategy for cancer cell detection on the basis of the recognition-induced aptamer conformation alteration and hybridization-induced fluorescence enhancement effect of DNA−AgNCs in the proximity of G-rich DNA sequences (Figure 4C). Target-induced fluorescence quenching is another detection strategy that uses AuNCs as fluorescence labels, and this approach has been employed for the determination of miRNA,[152] deoxyribonuclease I,[153] and protein kinase.[154] Dong *et al.*[155] reported an enhanced fluorescent sensor for miRNA, where the hybridization of target and MB can induce the Hg^{2+} ion-meditated conformational change in MB probe to form a Y-shaped junction structure leading to the release of Hg^{2+} in the presence of an assistant probe to cause fluorescence quenching of AgNCs (Figure 4D). Meanwhile, the formed Y-shaped junction structure can be efficiently cleaved by the endonuclease to trigger target recycling for signal amplification. Although AgNCs for fluorescent signal output have gained considerable attention in bioanalysis applications, their fluorescence is sensitive to the concentration of chloride anions due to the strong complexation between Ag(I) and Cl^-. In addition, AgNCs are prone to oxidation.

Apart from AuNCs and AgNCs, copper nanoclusters (CuNCs) have also been extensively used as promising fluorescence probes for detecting various targets, such as proteins,[156] single nucleotide polymorphisms (SNPs),[157] miRNAs,[158] cancer cells,[159] and so on. Jiang *et al.* recently reported on a sensitive fluorescent biosensor for the identification of SNPs on the basis of DNA-mediated *in situ* synthesis of CuNC, similar to AgNCs.[157] However, the developed monomeric CuNCs showed weak fluorescence and poor stability. To overcome this problem, Xu *et al.*[158] proposed a concatemeric dsDNA−CuNCs strategy by introducing the rolling circle replication technique in CuNP synthesis, which exhibited 4 orders of magnitude improvement in sensitivity. In addition, Sha and colleagues[156] integrated the *in situ* synthesis of AT-rich dsDNA−CuNCs as fluorescence reporters for hairpin DNA cascade signal amplification reaction to facilitate ultrasensitive quantification of transcription factor of nuclear factor-kappa B1 (p50). Moreover, through the introduction of enzyme-mediated substrate hydrolysis, target-induced *in situ* formation of DNA−CuNCs was also successfully employed for a variety of enzymes such as telomere,[160] DNA methyltransferase,[161] and nuclease.[162] Moreover, Zhang *et al.*[159] demonstrated a no-wash fluorescence biosensing platform for directly amplified measurement of human breast cancer cell line MCF-7 and miRNA-21 in solution with the combination of HCR and *in situ* synthesis of DNA−CuNCs.

NANOPARTICLES AS FLUORESCENCE QUENCHERS

Fluorescence quenching-based no-wash biosensors are one of the most important energy-transfer-based analytical platforms, where the fluorescence of the donor can be effectively quenched by the acceptor in the presence or absence of target analytes. The fluorescence of the donor is an on or off switch dependent on the presence of targets, in which the changes in fluorescence signal are relative to the concentration of the target. Recent reports have demonstrated highly efficient fluorescence quenching of the fluorescence of various fluorescence molecules in the presence of various nanomaterial quenchers, including AuNPs, carbon nanomaterials (*e.g.*, carbon nanotubes (CNTs), graphene oxide (GO), carbon nanoparticles (CNPs), and other nanoquenchers.[11,163,164] In these amplified quenching processes, the Stern−Volmer quenching constants (10^7−10^{10} M^{-1}) show 5−7 orders of magnitude improvement when compared to conventional fluorescence quenching processes (10^2 M^{-1}).[165] These super strong fluorescence quenching properties have been widely exploited to develop rapid and sensitive no-wash biosensing strategies for tumor biomarkers in solution.

AuNPs. AuNPs are excellent quenchers due to their high molar extinction coefficients and tunable absorption spectra in the visible region. When fluorescent molecules are close enough to the surface of AuNPs, AuNPs can quench the fluorescence by plasmonic resonance energy transfer. This advantageous property has stimulated extensive research using AuNP-induced fluorescence quenching of luminescent molecules covering

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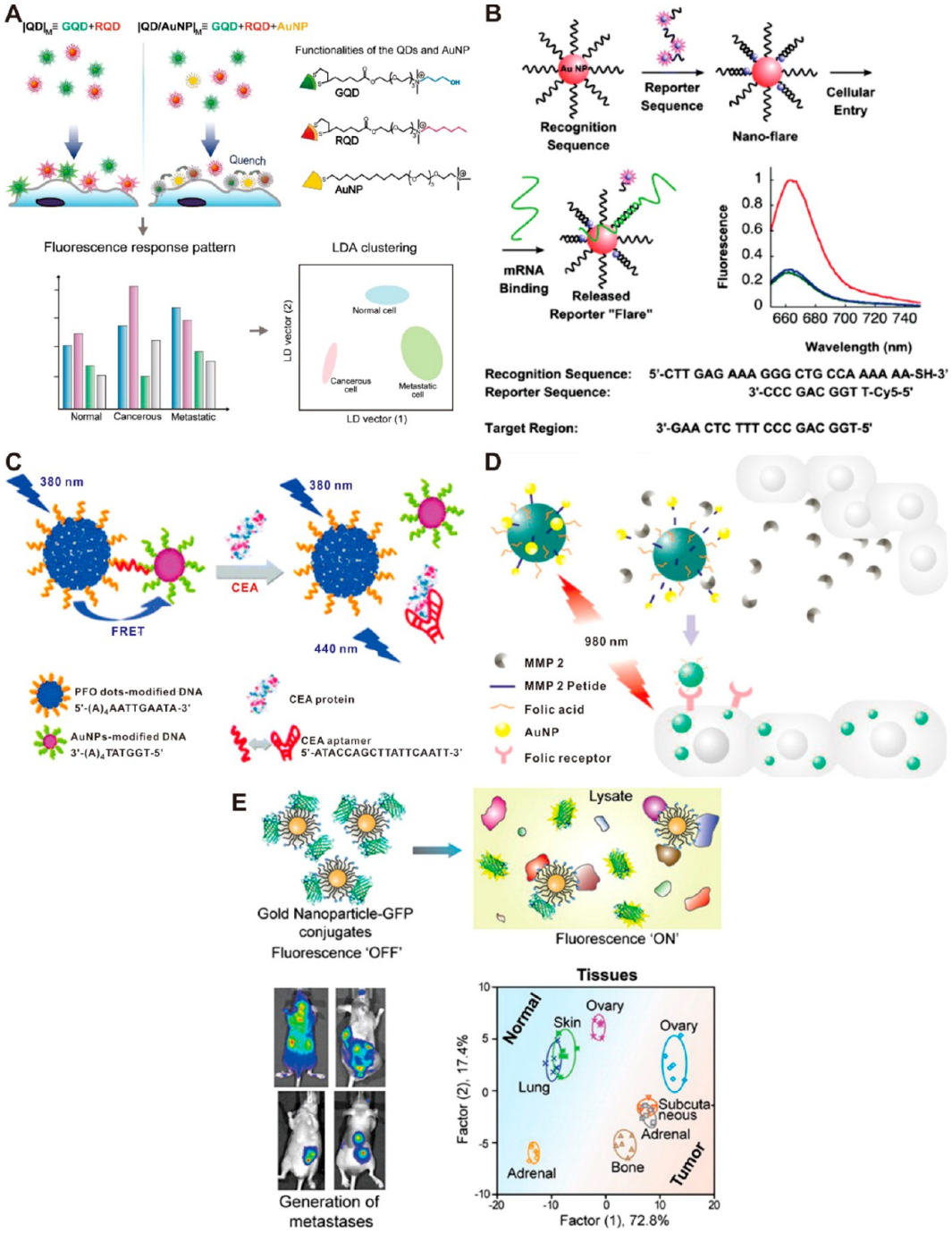

Figure 5. (A) The interaction between the nanoparticles and cell surface in the sensing system generating differential quenching and providing distinct patterns to discern different types/states of cells. Reprinted with permission from ref 171. Copyright 2012 Elsevier Ireland Ltd. (B) Nanoflares for mRNA detection in living cells. Reprinted with permission from ref 176. Copyright 2007 American Chemical Society. (C) FRET between PFO dots and AuNPs for CEA analysis. Reprinted with permission from ref 181. Copyright 2012 The Royal Society of Chemistry. (D) UCNP@p-Au sensed by MMP 2-expressing cancer cells. Reprinted with permission from ref 190. Copyright 2016 Elsevier B.V. (E) Schematic illustration of fluorescence modulation by the competitive binding between the quenched NP-GFP complex and the lysate proteins. Reprinted with permission from ref 193. Copyright 2012 American Chemical Society.

diverse fields of applications. In no-wash fluorescent biosensors, AuNP quenching-based biosensors mainly depend on two formats of target-induced fluorescence quenching and target-induced fluorescence recovery. In the first method, the fluorescent molecules are situated at a distance far away from the AuNP surface without target, resulting in high fluorescence, whereas the fluorescence decreases when AuNPs are in close proximity to fluorescent molecules in the presence of target molecules. This method has been extensively applied to detect various tumor markers, including DNAs,[166] proteins,[167,168] enzymes,[169,170] and even cancerous cells.[171] Wang *et al.*[166] reported DNA-induced fluorescence quenching for the detection of the p53 gene and SNP in solution by using gold nanorods (AuNRs) as a fluorescence quencher. Liu and co-workers[171] designed a fluorescent sensing method for the identification of mammalian cell types (*e.g.*, normal, metastatic,

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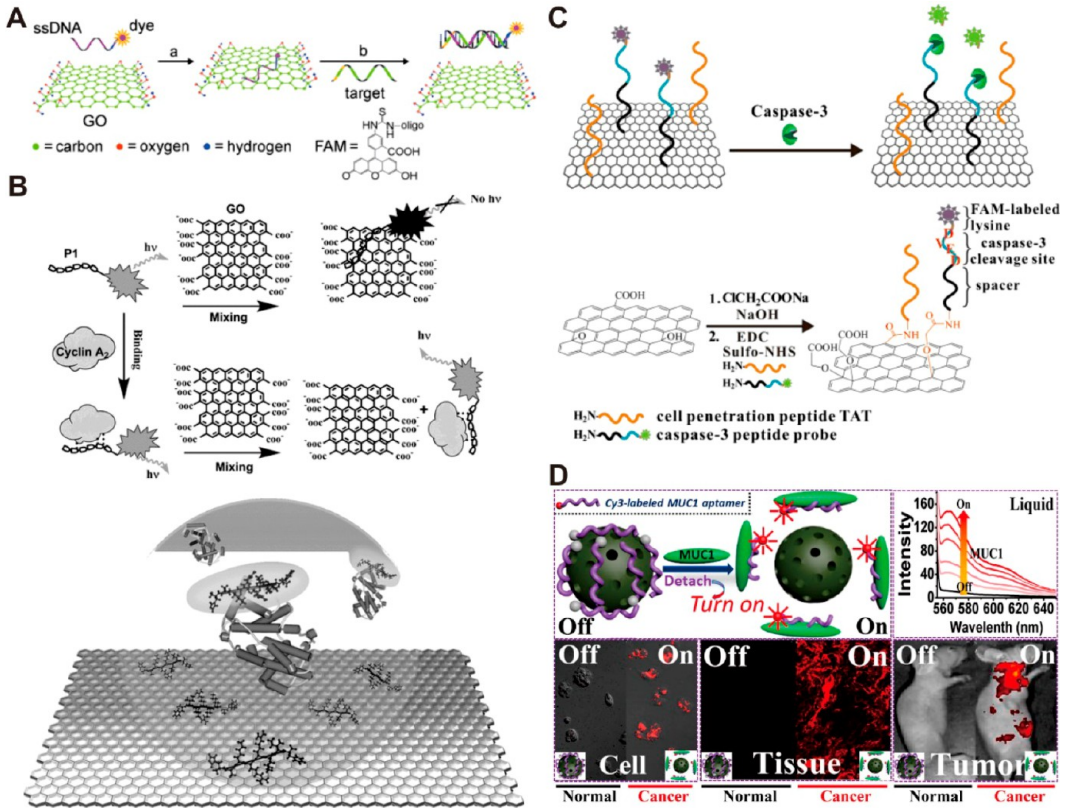

Figure 6. (A) Target-induced fluorescence change of the ssDNA−FAM−GO complex. Reprinted with permission from ref 202. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA. (B) No-wash biosensor for cyclin A2 by using preferential quenching of fluorescence from unbound P1 by GO. Reprinted with permission from ref 221. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA. (C) GO−peptide conjugates for caspase-3 detection. Reprinted with permission from ref 224. Copyright 2011 Wiley-VCH Verlag GmbH & Co. KGaA. (D) CNP/P0-Cy3 aptasensor for MUC1 protein and cancer cell. Reprinted with permission from ref 234. Copyright 2015 American Chemical Society.

and cancerous cells) using AuNPs as QD quenchers (Figure 5A). Unfortunately, without targets, a high fluorescence background could reduce the analytical sensitivity. Target-induced fluorescence recovery is achieved by pre-immobilizing the fluorescent materials onto the AuNP surface to minimize or eliminate background noise in the absence of targets. In contrast, with targets, the fluorescent molecules detach from the AuNP surface, thereby recovering the fluorescence. With this principle, researchers have fabricated various no-wash fluorescent biosensors for determining a wide range of analytes, like small molecules,[172−174] DNAs,[175] RNAs,[176] proteins, enzymes, as well as cells. Seferos *et al.*[176] first designed nanoflares for mRNA detection in living cells using AuNPs and cyanine 5 (Cy5) as quencher and flares, respectively (Figure 5B). Recently, a nanopyramid self-assembly composed of AuNPs and UCPs was reported for ultrasensitive detection of miRNA in living cells, where the presence of target miRNA leads to the complete dissociation of nanopyramid self-assemblies and consequently fluorescence signal recovery.[177] In the presence of miRNA, circular dichroism signal also diminishes, which can be used for quantifying target miRNA as well. Zhao *et al.*[178] constructed a DNA-driven core−satellite assembly of AuNPs and QDs for *in vitro* measurement of miRNA. Meanwhile, a similar core−satellite nanostructure was also fabricated for ultrasensitive detection of tumor protein markers of prostate-specific antigen (PSA)[179] and thrombin.[180] By employing target binding-induced conformational changes in aptamers to cause the fluorescence recovery of quenched dyes onto the AuNP surface, tumor protein biomarker CEA was successfully monitored (Figure 5C).[181] Combined with conventional immunoassay, AuNP-based no-wash fluorescence immunosensor has been explored to detect AFP.[182,183] In addition, AuNP-based fluorescence quenching coupling with MB can be used to monitor tumor-related telomerase[184] and matriptase.[185] Using enzyme-triggered cleavage of a peptide linker, AuNP-based fluorescent sensors could also be employed to measure caspase-3,[186−188] type IV collagenase,[189] and MMP-2.[190] Chan *et al.*[190] constructed a no-wash fluorescence biosensor for monitoring the MMP-2 activity by using AuNPs as quenchers for polypeptides conjugated to UCPs, where the target can catalytically hydrolyze the peptides at a special cleavage site that can cause the separation of AuNPs and UCPs, further giving rise to the fluorescence recovery (Figure 5D). Furthermore, AuNP-based fluorescent biosensors can be exploited to detect cancerous cells. An exciting design was demonstrated from Rotello and colleagues,[191−193] who described a series of array-based fluorescent biosensor systems for the identification of cell types based on fluorescent AuNP conjugates and cell-induced quenched fluorophore displacement, in which the subtle changes in the physicochemical nature of different cell surfaces can give rise to a significant fluorescence response (Figure 5E). Additionally, Halo *et al.*[194] employed AuNPs and Cy5 as nanoquencher and reporter flare respectively to design fluorescent nanoflares for the measurement of metastatic breast cancer cells, where the reporter flare

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is displaced after the binding of mRNA with the recognition sequence, yielding an increased fluorescent signal.

Carbon Nanomaterials. In recent years, numerous classes of carbon nanomaterials have been explored for potential applications in bioassays, due to their special optical, structural, chemical and electronic properties.[195] On the basis of their excellent fluorescence quenching abilities, carbon nanomaterials such as graphene and its derivatives, CNTs and CNPs, can serve as efficient nanoquenchers in a no-wash fluorescent sensor system.[196] Compared with organic quenchers, carbon nanomaterials have shown superior quenching efficiency for a variety of fluorophores, with low background and high signal-to-noise ratio. Accordingly, they have been extensively investigated for sensing applications.[196] Previous study demonstrated that the sp^2 carbon atoms on the surface of carbon nanomaterials can interact strongly with ssDNA through $\pi-\pi$ stacking interaction, which can efficiently quench the fluorescence of dye-tagged ssDNA probes.[195,197,198] On the contrary, the dye-labeled dsDNA retains strong fluorescence since carbon nanomaterials do not interact with the rigid structure of the produced dsDNA or aptamer-target complexes due to efficient shielding of nucleobases within the negatively charged dsDNA phosphate backbone. Thus, a simple, sensitive, and versatile fluorescent quenching-based biosensor can be developed by using carbon nanomaterials as nanoquenchers. Yang and co-workers[199] developed a no-wash fluorescent biosensor for specific DNA based on the assembly of CNTs and FAM-labeled ssDNA. Meanwhile, this team also reported a similar fluorescence biosensor with the combination of MBs, and the study concluded that this sensor largely improves the signal-to-background ratio and only needs one labeled fluorophore compared with conventional MBs.[200] Subsequently, based on a similar strategy, many no-wash fluorescent nanosensors for DNA detection were reported by the self-assembly of various fluorophore-conjugated probes and CNTs.[201] Similar to CNT-based fluorescence quenching biosensors, Lu *et al.*[202] proposed a GO-based no-wash biosensing platform for DNA detection through the strong noncovalent binding between dye-labeled ssDNA and GO (Figure 6A). After that, Balapanuru *et al.*[203] designed an interesting GO−organic dye ionic complex (PNP^+GO^-) based on the noncovalent electrostatic adsorption interactions of 4-(1-pyrenyl-vinyl)-*N*-butylpyridinium bromide and GO and found that DNA exhibits a greater ionic attraction for PNP^+ than the weakly ionizable GO. Therefore, the PNP^+GO^- complex can be used to selectively determine DNA. Besides, using GO as nanoquencher can increase the sensitivity of conventional MBs.[204−206] Dong and colleagues[206] designed a fluorescent sensor for sensitive DNA detection *via* GO-enhanced MB. To further increase the sensitivity, Peng *et al.*[207] proposed an amplified GO quenching-based DNA detection strategy with the help of exonuclease III-induced target recycling. Besides CNTs and GO, CNPs, possessing broad light-absorption windows, can also function as a nanoquencher for no-wash fluorescent biosensors because dye-labeled ssDNA can also adsorb onto the CNP surface *via* $\pi-\pi$ interaction. Recently, many studies have focused on CNP-based fluorescence quenching as a no-wash sensing platform for DNA detection.[208−210] In addition, carbon nanomaterial-based no-wash fluorescent nanosenors for miRNA assay have been widely developed in recent years using the same principle as DNA detection. Lu and co-workers[211] first developed a GO-based no-wash fluorescence quenching approach for rapid detection of miRNA. Cai *et al.*[212] integrated the site-specific cleavage of the endonuclease with GO-based fluorescence quenching for sensitive no-wash detection of miRNA. Recently, some highly sensitive and accurate no-wash detection sensors for miRNA were described by coupling GO-based fluorescence quenching with signal amplification methods, such as HCR,[213] circular exponential amplification,[214] and toehold-mediated nonenzymatic amplification.[215] Apart from GO, CNPs were also applied for no-wash miRNA assay.[216]

Carbon nanomaterial-based no-wash fluorescent nanosensors have played a key role in tumor protein biomarker detection. Lu *et al.*[202] first fabricated a GO-based fluorescence aptasensor for no-wash detection of thrombin, where FAM-tagged thrombin aptamers that strongly bind with GO were used as recognition molecules and signal outputs. After that, several similar no-wash aptasensors were reported for thrombin[217,218] and CEA[219] detection. Recently, an interesting design in this field was described by Liu *et al.*,[220] who combined a GO-based biosensing platform with a DNA probe release and rolling circle amplification method for ultrasensitive detection of thrombin. Like aptamers, peptides can quickly adsorb onto the surface of carbon nanomaterials. Wang *et al.*[221] used dye-labeled peptides as recognition molecules and signal outputs for development of a no-wash detection system for cyclin A_2 based on GO and CNTs as nanoquenchers, respectively (Figure 6B). In this case, the authors found that GO-based no-wash biosensor presented a higher sensitivity than that of CNT-based biosensors for cyclin A_2 detection. Combined with MB-like probes, GO-based fluorescence biosensors can likewise detect protein biomarkers in solution.[222] Immunoassay is one of the most popular methods in protein detection. Through the combination of conventional immunoassay, Liu and co-workers[223] designed a GO-based no-wash fluorescence immunosensor to measure AFP. As previously mentioned, peptides have strong binding affinity with carbon nanomaterials. Using dye-labeled peptides as hydrolytic substrates, carbon nanomaterial-based fluorescence quenching biosensors can be used to monitor tumor-related enzyme activity. Wang *et al.*[224] conjugated FAM-tagged peptide substrates with GO for sensing intracellular protease, caspase-3 (Figure 6C). Several similar reports were published on the detection of enzyme biomarkers, such as MMP-2,[225−228] thrombin,[229] as well as tyrosine phosphatase.[230] Moreover, coupled with site-specific cleavage, a GO-based no-wash fluorescence platform was also employed for monitoring endonuclease[231] and methyltransferase.[232] Carbon nanomaterial-based no-wash fluorescent nanosensors are extremely attractive for direct quantitative assay of cancer cells. Cao and colleagues[233] developed a GO-based fluorescent biosensor for *in vitro* detection of CCRF-CEM cancer cells through a FAM-labeled aptamer−GO complex. Recently, Li and co-workers[234] constructed a fluorescent aptasensor for *in vitro* detection of MUC1 protein and MCF-7 tumor cells in solution by using oxidized mesoporous CNPs as fluorescence quenchers for Cy3-labeled aptamer probes (Figure 6D).

Other Nanoquenchers. Although AuNPs and carbon nanomaterials have become the most common nanoquenchers for fluorescence biosensors, an increasing number of nanomaterials, such as graphene-like 2D layered nanomaterials and MOFs, have attracted great interest as fluorescence quenchers in fluorescence quenching sensors in recent years. Graphene-like 2D layered nanomaterials, including graphite-carbon nitride ($g\text{-}C_3N_4$) nanosheets, and various layered transition-metal dichalcognides (TMDs) have been investigated for fluorescence

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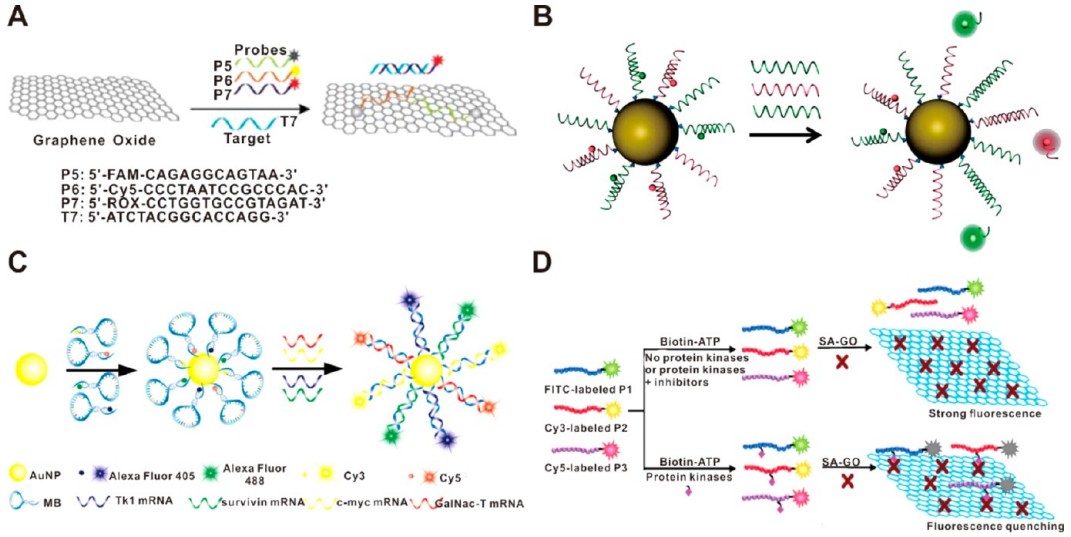

Figure 7. (A) GO-based multicolor DNA analysis. Reprinted with permission from ref 262. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA. (B) Multiplexed nanoflares for simultaneous detection of target mRNA. Reprinted with permission from ref 271. Copyright 2012 American Chemical Society. (C) Four-color nanoscale MB for detection of multiple intracellular mRNAs. Reprinted with permission from ref 274. Copyright 2013 American Chemical Society. (D) Multicolor fluorescent GO nanosensor for the multiplex detection of protein kinase activity and inhibition. Reprinted with permission from ref 276. Copyright 2016 Elsevier B.V.

quenching-based biosensors because of their structural similarities to graphene and similar 2D structures and excellent optical properties.[197,235] For example, a g-C_3N_4 nanosheet can quench fluorophores *via* photoexcited electrons transfer. Wang *et al.*[236] described a no-wash fluorescence biosensor for DNA detection with g-C_3N_4 nanosheets to quench FAM-labeled ssDNA. Thereafter, Liao *et al.*[237] developed a multifunctional g-C_3N_4 nanosheet-based fluorescence quenching platform for intracellular miRNA detection. Layered TMDs have also been widely verified as promising fluorescence quenchers in constructing fluorescence quenching-based sensing platforms because of their excellent light absorption capacity and fast electron-transfer rate. Recently, various nanosheets, such as MoS_2,[238−240] WS_2,[241] TiS_2,[242] TaS_2,[242] CoS_2,[243] and Ta_2NiS_5,[244] have been employed for no-wash sensing platforms for target DNA detection on the basis of their quenching abilities toward fluorescently labeled ssDNA. In these no-wash biosensing systems, the fluorescently labeled ssDNA was adsorbed on the surface of layered TMDs through van der Waals interaction between nucleobases and the basal plane of TMDs that cause fluorescence quenching of labeled ssDNA. Additionally, Xi *et al.*[245] presented a sensitive fluorescence quenching-based detection method for miRNA by using WS_2 nanosheets as a nanoquencher with DSN signal amplification. Moreover, fluorescence biosensors for protein biomarker detection have been developed by using fluorescence-tagged aptamers as probe and layered TMD as a fluorescence quencher, respectively. By controlling the association and dissociation of fluorescence-tagged aptamers from layered TMD surfaces, layered TMD-based no-wash fluorescence sensors can detect different cancer protein biomarkers, including PSA,[246] CEA,[247] and epithelial cell adhesion molecule (EpCAM).[248] Furthermore, layered TMD-mediated fluorescence quenching strategy has also been described for the detection of enzymatic activities and their inhibitors based on enzyme-catalyzed substrate hydrolysis. Recently, this strategy was used to monitor a variety of enzymes, such as DNA methyltransferase,[249] nuclease,[250] as well as T4 polynucleotide kinase.[251]

MOFs are a class of crystalline porous materials constructed by assembly of metal ions or metal-containing clusters with organic linkers. MOFs exhibit ultrahigh flexible porosity, tremendous surface areas, and well-defined structures, which have been widely applied in gas storage, chromatographic separation, heterogeneous catalysis, as well as drug delivery and release.[252,253] Recently, Liu *et al.*[254] used MOFs as a quencher for no-wash fluorescence detection of nucleic acids. Like carbon nanomaterials, the MOF can also adsorb dye-labeled ssDNA *via* $\pi-\pi$ stacking interactions or hydrogen-bond interactions, which strongly quench the dye fluorescence; whereas the resultant dsDNA from target DNA hybridization detaches from the MOF surface, thus leading to fluorescence recovery. In following, MOFs as fluorescence quenching platforms for DNA detection have been widely investigated.[255−258] Additionally, Zhu and colleagues[259] first demonstrated a MOF-based fluorescence quenching biosensor for target protein thrombin detection.

Fluorescence Quenching-Based Multiplexed Biosensors. Although various sensitive and selective analytical methods have been widely reported for the detection of single biomarkers, they remain inadequate for clinical diagnosis of diseases with a high heterogeneity like cancer owing to the insufficient signal resolution.[260] The combination of multiple biomolecules would be expected to be more effective for improving disease diagnostics than only concentrating on a single biomarker.[261] Thus, exploring highly sensitive multiplexed assays that can specifically target several target analytes in parallel within a single sample is very attractive for the early diagnosis of cancer. Additionally, multiplexed biosensors for simultaneous detection of biomarkers could largely decrease total analytical time and sample volume. To date, multiplexed and simultaneous detection of biomarkers including nucleic acids, enzymes, or proteins has been employed to enhance *in vitro* early diagnostics of cancer and monitor the response to treatment. Among these biosensors, fluorescence quenching-

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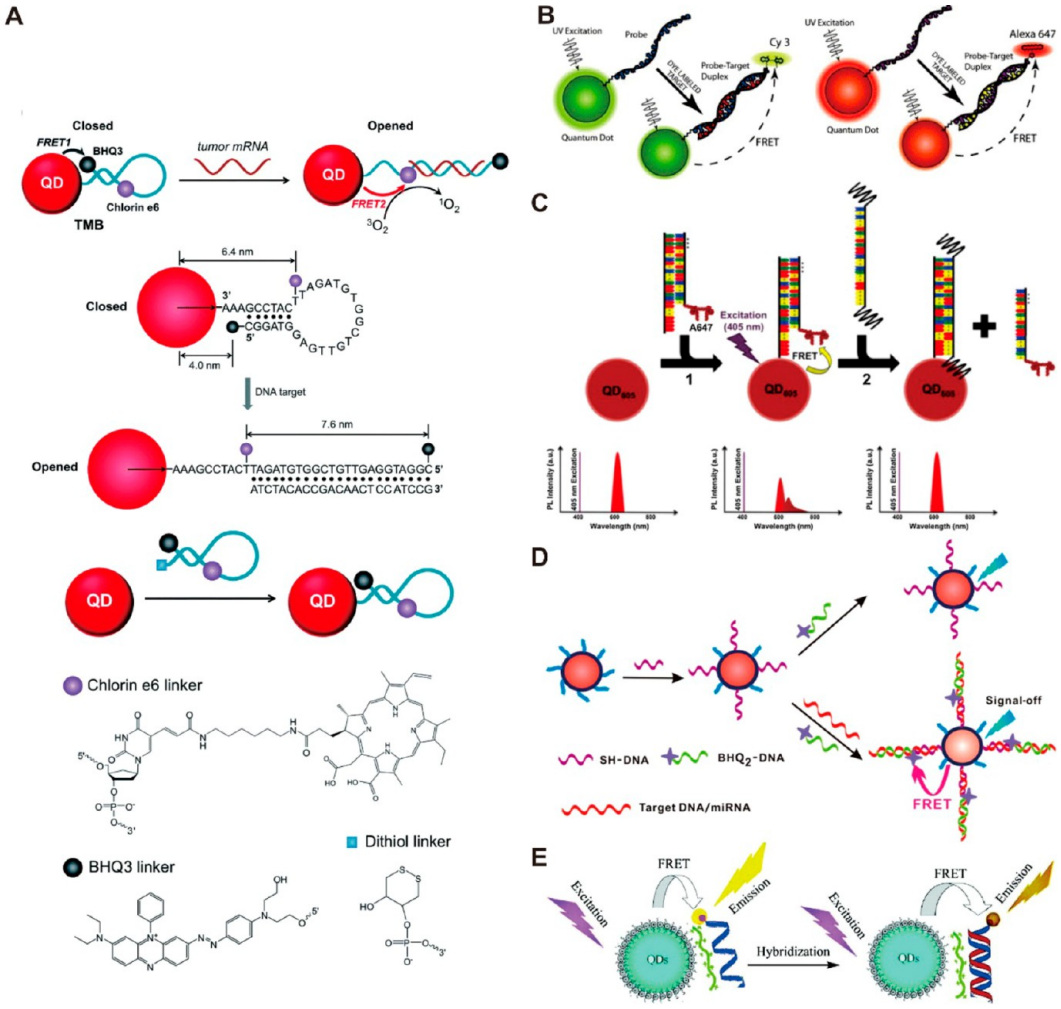

Figure 8. (A) 2D MB for tumor overexpressing target mRNA molecules. Reprinted with permission from ref 286. Copyright 2015 The Royal Society of Chemistry. (B) QD-FRET-based strategy for two-color nucleic acid detection. Reprinted with permission from ref 288. Copyright 2006 Elsevier B.V. (C) Competitive displacement assay utilizing QD as a FRET donor. Reprinted with permission from ref 290. Copyright 2012 Elsevier B.V. (D) Sandwich FRET nanosensors for detection of DNA/miRNA. Reprinted with permission from ref 289. Copyright 2013 American Chemical Society. (E) FRET detection system for target DNA detection based on electrostatic adsorption. Reprinted with permission from ref 280. Copyright 2007 American Chemical Society.

based multiplexed no-wash biosensors using nanomaterials as nanoquenchers have played a pivotal role. Fluorescence quenching-based multiplexing DNA biosensors are achieved by combining different nanomaterial−ssDNA interactions to construct multicolor fluorescent DNA nanoprobes or by using nanomaterials to fabricate multicolor nanoscale MBs. He *et al.*[262] developed a multicolor fluorescence quenching-based strategy for no-wash detection of target DNA by employing GO−ssDNA interactions (Figure 7A). However, it has been reported that DNA molecules are prone to nonspecifically adsorb to GO surfaces. By combining the traditional MB, Song *et al.*[263] reported on a AuNP-based multicolor nanoscale MB for no-wash detection of multiple tumor DNA markers in solution by the self-assembly of multiple hairpin probes labeled with different fluorophores and AuNPs. Subsequently, CNT- and MOF-based multicolor nanoscale MBs were also reported for multiplexed DNA detection.[264,265] Nevertheless, these nanoscale MBs generally suffer from poor salt and thermal stability or poor detection specificity. To address this problem, Su *et al.*[266] designed AuNP-decorated silicon nanowires with robust salt and thermal stability and high quenching efficiency as superquenchers to construct multicolor fluorescent nanoscale MBs for multiplexed no-wash analysis of DNA in solution. Similar to multiple DNA detection, the multiplexing detection of miRNA was also achieved by exploiting interactions between nanomaterials and ssDNA molecules to construct multicolor fluorescent DNA nanoprobes. Dong *et al.*[267] described a highly sensitive and no-wash multiple miRNA detection method through the strong GO−ssDNA interaction to establish multicolor fluorescence probes. A similar study was also conducted for a multiplexed quantitative biosensor of miRNA in living cells.[268] More recently, other nanomaterials, such as g-C_3N_4 nanosheets[269] and MOFs,[270] have also been utilized as nanoquenchers for fluorescence-labeled ssDNA or peptide nucleic acids for no-wash simultaneous detection of multiplexed miRNA *in vitro*. Besides miRNA, mRNA (mRNA) has also been widely applied as an important tumor marker for the diagnosis and treatment of cancer cells. However, determining a single mRNA biomarker may lead to false-positive results. Two possible reasons are that cancers are associated with bulk expression of multiple mRNA markers and some mRNAs are overexpressed in normal cells. Therefore, simultaneous

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detection of multiple mRNA biomarkers in living cells is of great importance to improve the accuracy of early cancer diagnosis. Prigodich and colleagues[271] designed multiplexed fluorescence AuNP-based nanoflares for simultaneous detection of two distinct mRNA targets in a living cell (Figure 7B). Li *et al.*[272] successfully used a similar multicolor fluorescence nanoprobe for the simultaneous detection of three intracellular tumor-related mRNAs. Moreover, combined with fluorescence quenching and traditional MBs, Pan *et al.*[273,274] demonstrated a AuNP-based multicolor nanoscale MB for simultaneous detection of multiple mRNA markers in living cells (Figure 7C).

Tumors may be detected with multiplexed protein kinase biomarkers. Lee *et al.*[275] provided a fluorescence quenching-based multiplexed no-wash biosensor for simultaneous detection of protein kinases using titanium dioxide (TiO_2)-decorated GO, in which TiO_2 and GO were employed to adsorb multicolor dye-labeled phosphorylated peptides and quench these dyes' fluorescence, respectively. A similar GO-based no-wash multiplexed fluorescence biosesnsor was presented for multiplexed protein kinase detection in solution *via* enzyme-catalyzed phosphorylation-induced self-assembly of multicolor peptide probes onto GO (Figure 7D).[276]

NANOPARTICLES AS FLUORESCENT NONQUENCHERS

QD-Based Förster Resonance Energy Transfer Biosensors. Due to their special optical properties over organic dyes, QDs, as a promising fluorescent probe, have been frequently used in a large variety of *in vitro* bioassays.[277] Especially, QD-based Förster resonance energy transfer (FRET) biosensors have attracted significant interest because they can directly measure the presence of target analytes in solution, thereby effectively avoiding the problems of long incubation times, multiple wash steps, and the conformation of recognition molecules upon immobilization. The past few decades have witnessed the rapid development of QD-based FRET biosensors for detecting various tumor markers in solution.[278] In FRET biosensors, QDs can function as donors, acceptors, or even both at the same time.

Compared with traditional organic dyes, QDs as donors in FRET applications can provide many advantages, such as a better spectral overlap with different acceptors, a higher FRET efficiency because of higher acceptor density on the QD surface, and a wider excitation wavelength range of QD donors to avoid overlap of acceptor excitation. Willard *et al.*[279] first pioneered a QD donor-based FRET to a dye-labeled biomolecule. After that, using QDs as FRET donors in different sensing strategies was described in the literature for the determination of nucleic acids,[280] proteins,[281] enzymes,[282] as well as small molecules.[283,284] Nucleic acids, such as DNAs and RNAs are very important biomarkers for sensing applications in early detection of cancers. To develop QD-based FRET sensing for nucleic acid detection, target-induced hybridization with complementary sequences has been exploited to adjust the FRET efficiency. Recently, several target-induced hybridization-based FRET biosensing formats have been explored by the adoption of QD donors, including MBs and hybridization probes to detect targets based on sandwich, proximity, and displacement assays, and nonspecific electrostatic adsorption. Kim *et al.*[285] first represented a hybridization-based FRET biosensor using QD-conjugated MBs for target DNA, where the addition of target would result in the decrease of FRET efficiency owing to the presence of opened hairpin. Another interesting study was described by Ma and co-workers, who designed a QD-modified 2D MB by integrating a photosensitizer (chlorin e6), QD, and a dark quencher (BHQ3) into a hairpin DNA molecule to generate multiple synergistic FRET pathways, in which QD-modified 2D MB could be activated by tumor overexpressing target mRNA molecules (Figure 8A).[286] Although the conformational changes of MB can modulate the FRET response, hybridization probes on the basis of proximity, displacement, and sandwich assays have also been used to adjust the FRET using QD as donors. Bakalova *et al.*[287] reported a QD-based FRET sensor for target mRNA by the hybridization of small-interfering RNA-conjugated QDs and target mRNA amplified by Cy5-labeled nucleotides. Subsequently, Algar and Krull[288] designed a multiplexed hybridization-based FRET sensor for simultaneous detection of two target DNAs in solution based on the hybridization proximity between QD-oligonucleotide conjugates and dye-labeled targets, where two colored CdSe/ZnS QDs and two dyes (Cy3 and Alexa647) acted as FRET donors and acceptors, respectively (Figure 8B). In addition, sandwich and competitive displacement hybridization-based FRET biosensors for no-wash detection of target DNA have been explored. The former requires a capture probe (oligonucleotide-conjugated QD donor) and a detection probe (oligonucleotide-labeled acceptors) to hybridize with the help of target DNA, thereby producing an effective FRET assay; whereas the later depends on the higher stability of the probe-target DNA hybrid complex than that of a partial complementary, mismatch, or shorter-length sequence to dye-labeled probe that can facilitate energetically favorable displacement reaction. Two typical examples from Su *et al.*[289] and Vannoy *et al.*[290] demonstrated sandwich hybridization and competitive displacement hybridization-based FRET in the application of *in vitro* DNA or RNA detection, respectively (Figure 8C and 8D). Moreover, a single QD-based FRET nanosensor in a separation-free format from Zhang *et al.*[291] was constructed for ultrasensitive detection of target DNA by using a sandwich hybridization of Cy5-labeled capture probes, biotinylated detection probes, target DNA, and streptavidin-coated QD to form a FRET donor−acceptor ensemble. Two similar examples were displayed later for analysis of DNA point mutations related to human epidermal growth factor receptor (EGFR) gene[292] and for quantitative determination of miRNA by coupling with an exponential amplification reaction,[293] respectively. On this premise, Zhang *et al.*[294] explored a multiplexed single QD-based FRET sensor for simultaneous detection of two target DNAs, in which QD were cohybridized with A488- and A647-labeled detection probes through sandwich hybridization. Another no-wash QD-based FRET sensor for target DNA or RNA detection was achieved by using nonspecific electrostatic assembly between negatively charged QD donors and dye-labeled ssDNA acceptors *via* a cationic polymer serving as a linker, in which with target DNA, the DNA duplex structure (dsDNA), exhibits larger rigidity and negative charge compared with ssDNA and can cause an increased distance between QDs and dsDNA, thus reducing the FRET efficiency. Peng and colleagues[280] first demonstrated the feasibility of this method (Figure 8E), and then several improved no-wash QD-based FRET biosensors were reported for target DNA detection.[295−297] While target-induced hybridization-based FRET nanosensors have obtained great success in the detection of nucleic acids, target-induced aptamer structural changes have also been used to measure cancer protein biomarkers using FRET with QD as donors.

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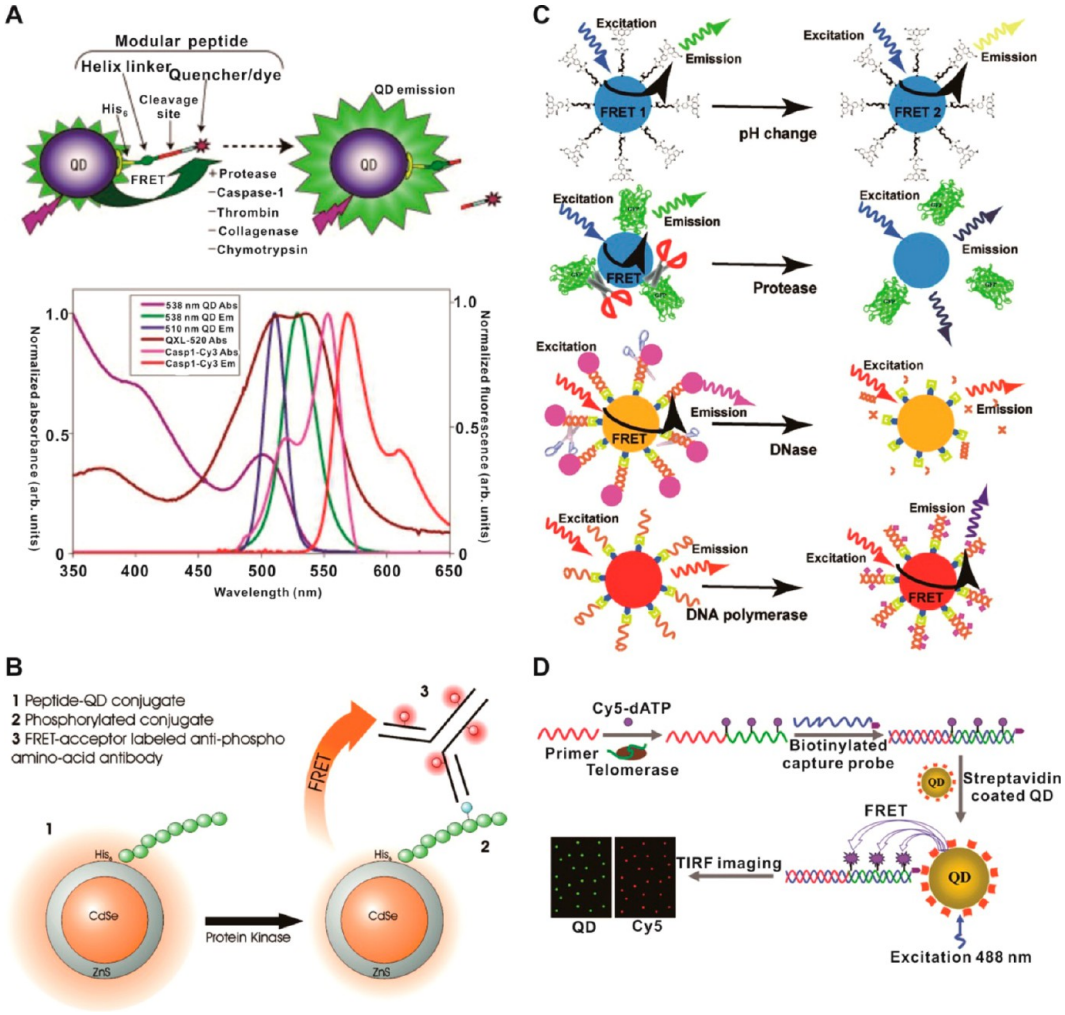

Figure 9. (A) Self-assembled QD-peptide nanosensors for protease activities. Reprinted with permission from ref 303. Copyright 2006 Nature Publishing Group. (B) Kinase-mediated phosphorylation of peptide-QD conjugates, antibody recognition of phosphopeptide, and FRET detection. Reprinted with permission from ref 310. Copyright 2010 American Chemical Society. (C) FRET-based QD bioprobes designed to trigger FRET changes by (from top to bottom): pH change, cleavage of a GFP variant with an inserted sequence recognized by a protease, digestion by DNase, and incorporation of fluorescently labeled dUTPs into ssDNA on a QD by extension with DNA polymerase. Reprinted with permission from ref 313. Copyright 2008 American Chemical Society. (D) Single QD-based biosensor for telomerase assay. Reprinted with permission from ref 314. Copyright 2015 The Royal Society of Chemistry.

Levy and co-workers[281] first pioneered a QD-based FRET biosensor for thrombin detection, where the presence of target protein can cause a substantial conformational change of the aptamer conjugated onto the QD surface to displace an oligonucleotide quencher conjugate, thereby modulating FRET efficiency. A similar example was described recently by Chi *et al.*[298] for measuring thrombin, where the strong interaction of thrombin and aptamer beacon-modified QD gave rise to the complete removal of dye BOBO-3 acceptor from the beacon owing to target-induced aptamer folding. The aptamer structure switching-based FRET sensors have also been developed for determining other tumor protein biomarkers, such as platelet-derived growth factor (PDGF),[299] MUC1,[300] and VEGF.[301] In addition, QD-based Bi-FRET aptasensor was designed for *in vitro* imaging detection of cancer cells based on QD−aptamer−doxorubicin (DOX) conjugates, where the QD fluorescence is quenched by DOX, while simultaneously DOX fluorescence is quenched by intercalation within the aptamer.[302] After targeting prostate cancer cells, DOX is gradually released from the conjugate to induce the fluorescence recovery of QD and DOX, thus achieving the imaging of cancer cells *in vitro*. No-wash FRET sensors using QD as donors have emerged as an alternative to monitoring the change of tumor-related enzyme expression levels due to its simplicity, accuracy, and sensitivity. QD-based FRET sensors targeted to enzyme activity have been utilized in several methods. The most widely exploited strategy relies on enzyme-catalyzed hydrolysis of acceptor dye-labeled substrate peptides pro-immobilized onto a QD surface. Because acceptor dye-labeled substrate peptides are in close proximity to QD donors, an efficient FRET between acceptors and donors is favored. With the target enzymes, the peptide substrates are cleaved by target enzymes, thus disrupting QD donor-dye acceptor FRET to provide signal transduction of enzymatic activity. The first demonstration of QD-based FRET sensors for monitoring protease activities was provided by Medintz *et al.*,[303] in which different dye-labeled peptide substrates were conjugated onto the QD surface by self-assembly to target different enzymes, including caspase-1, thrombin, collagenase, and chymotrypsin (Figure 9A). After that, several similar versions were explored for targeting other

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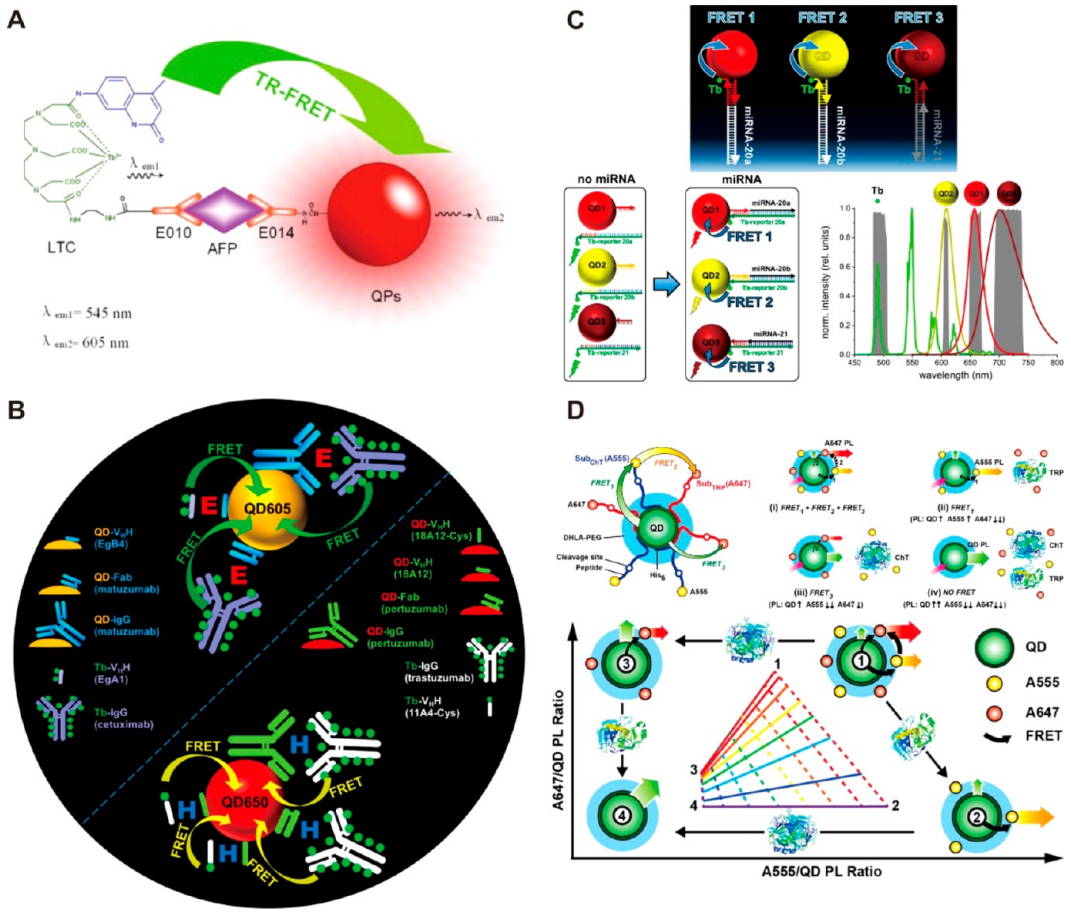

Figure 10. (A) QD-based double-antibody sandwich-type no-wash time-resolved fluoroimmunosensor for AFP detection. Reprinted with permission from ref 318. Copyright 2012 Elsevier B.V. (B) FRET sandwich immunosensors against EGFR and HER2. Reprinted with permission from ref 324. Copyright 2016 American Chemical Society. (C) Multiplexed miRNA assay with three different QDs (QD1, 2, 3) as acceptors. Reprinted with permission from ref 325. Copyright 2015 American Chemical Society. (D) Concentric FRET relay and three possible energy-transfer pathways between the QD, A555, and A647. Reprinted with permission from ref 328. Copyright 2012 American Chemical Society.

cancer-related enzyme biomarkers, such as MMPs,[304] trypsins,[305] and caspase 3.[306,307] Recently, an improved no-wash QD-FRET sensor for protein kinase assay was reported by Shiosaki *et al.*,[308] who found that the negatively charged QDs could recognize the change in net charge of the peptide upon phosphorylation, thereby causing a FRET response change. More recently, to broaden the further utility of peptide-based FRET sensors, Palomo *et al.* incorporated 3,4-dihydroxyphenylalanine (DOPA) into the peptide sequence to enable the quenching of QD through an electron-transfer mechanism in detecting aminopeptidase activity.[309] Compared with conventional peptide, the incorporation of DOPA conferred several obvious advantages, such as minimal structural changes, and the possibility for introduction at multiple positions within a biologically active peptide substrate. Another strategy for QD-based FRET to target enzyme activities depends on target enzyme-catalyzed phosphorylation of the peptide-QD conjugates to specifically recognize acceptor-labeled antiphosphotyrosine antibody, thereby resulting in the generation of FRET between QD donors and acceptor molecules. Ghadiali and coworkers[310] first performed the proof-of-concept validation of this method. The peptide substrates modified on the QD surface were first phosphorylated by tyrosine kinases in the presence of excess ATP, and then the phosphorylated products were detected by adding a specific Alexa Fluor 647-tagged antiphosphotyrosine antibody (Figure 9B). Later, a similar QD-based no-wash FRET nanosensor was developed to detect histone acetyltransferase activity.[311] Recently, Lim *et al.*[312] presented an interesting QD-FRET biosensor for measuring protein kinase activity, where Zn(II) rather than specific antibody was used to drive the occurrence of FRET between the phosphorylated acceptor 5(6)-carboxytetramethylrhodamine-labeled peptide and QD donors due to Zn(II)-mediated metal coordination. The third strategy for the no-wash detection of enzyme activity using QD-based FRET sensor rests upon the direct proteolytic or nucleolytic cleavage of acceptor dye-conjugated enzyme substrates from the QD surface with target enzymes. A representative example was demonstrated by Suzuki *et al.* (Figure 9C).[313] In this case, four different activated probes, including nuclease-activated QD-DNA conjugate FRET probe, proteolysis-activated QD-GFP FRET probe, ligation-based QD-labeled nucleotide FRET probe, as well as pH-switched fluoresceinyl-QD FRET probe, were designed for detecting protease, deoxyribonuclease, DNA polymerase, or pH changes. Moreover, the authors also found that this QD-based FRET sensor exhibited a multiplex detection capacity for a protease, trypsin, in the presence of deoxyribonuclease. Finally, a single QD-based FRET biosensor for the sensitive detection of telomerase activity was constructed by the hybridization of telomerase-induced Cy5-

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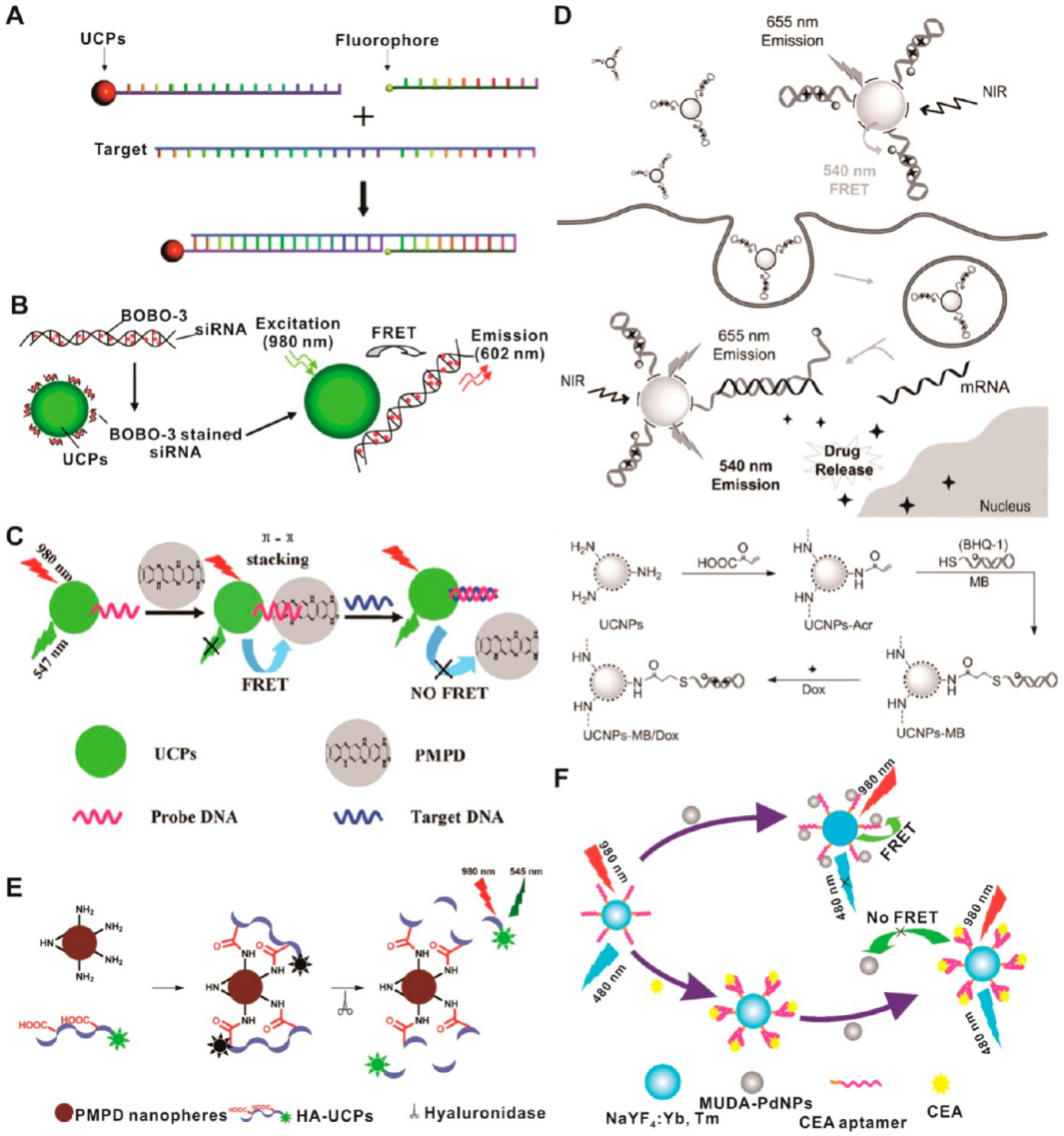

Figure 11. (A) UC-FRET-based nucleotide sensor through sandwich hybridization. Reprinted with permission from ref 338. Copyright 2006 American Chemical Society. (B) UC-FRET-based UCP/siRNA-BOBO3 complex system. Reprinted with permission from ref 340. Copyright 2010 American Chemical Society. (C) UC-FRET sensor with UCPs and PMPD nanospheres as the energy donor−acceptor pair. Reprinted with permission from ref 343. Copyright 2013 American Chemical Society. (D) UCP-based nanobeacons for tumor mRNA ratiometric detection and monitoring of drug delivery. Reprinted with permission from ref 346. Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA. (E) UC-FRET nanosensor for hyaluronidase using HA-UCNPs with PMPD nanospheres. Reprinted with permission from ref 348. Copyright 2015 American Chemical Society. (F) FRET biosensor for CEA based on CEA aptamer-attached UCPs to PdNPs. Reprinted with permission from ref 350. Copyright 2016 Elsevier B.V.

labeled primer with a biotinylated capture probe to form a biotinylated Cy5-labeled dsDNA. The resulting biotinylated Cy5-labeled dsDNAs could combine with streptavidin-coated QDs to produce a FRET pair using QDs as donors and Cy5 as acceptor molecules (Figure 9D).[314] Multiplexed detection of enzyme markers is vital to provide a more accurate clinical diagnosis of cancer. Lowe *et al.*[315] reported a multiplex QD-based FRET sensor of urokinase-type plasminogen activator (uPA) and human EGFR 2 enzymatic activities by synthesizing different enzyme-specific peptide substrates to prepare AuNP-biotinylated peptide conjugates or QD-His-tagged peptide conjugates *via* orthogonal coupling. Then, the AuNP-biotinylated peptide conjugates specifically bound to the streptavidin-coated QD surface, and the QD-His-tagged peptide conjugates bound to the Alexa Fluor 660-labeled antiphosphotyrosine antibody, thus producing two efficient FRET pairs of AuNP-QD and QD-Alexa Fluor for the detection of target enzyme activity. Subsequently, based on a similar format, two groups, Petryayeva *et al.*[316] and Chung *et al.*,[317] developed multiplexed no-wash FRET nanosensors using QDs as donors for simultaneously monitoring multiple cancer-associated enzyme biomarkers in solution.

Although FRET sensors using QDs as donors have developed into the most popular no-wash fluorescent biosensing platform, FRET sensing using QDs as acceptors has also gradually become a well-established analytical technology. Among these QD acceptor-based FRET biosensors, lanthanide-to-QD FRET using lanthanide as donors and QDs as acceptor molecules has gained considerable interest due to the capacity for complete suppression of light-excited

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QD luminescence through time-gated formats or the complete avoidance of QD light-absorption by using lanthanide NIR-excitation *via* photon up-conversion. Using the luminescent terbium complex as a FRET donor to fabricate QD-based no-wash fluorescent sensors has been reported for measuring various markers, like DNAs, RNAs, and proteins. Combined with conventional immunoassay, a no-wash Tb-to-QD FRET immunosensor (Figure 10A) has been employed for rapid and sensitive detection of protein biomarkers, including AFP[318] and CEA[319] in solution. Nevertheless, owing to large sizes of traditional antibodies that increase the donor−acceptor distance, a low FRET efficiency between the donors and acceptors would cause poor detection sensitivity. Thus, to improve the FRET efficiency, some related small biorecognition molecules, such as $F(ab')_2$, F(ab), and single-domain antibodies, were introduced to enhance the detection sensitivity of PSA,[320−322] EGFR,[323] and HER2.[324] Moreover, this Tb-to-QD FRET nanosensor also displayed the capacity of multiplexing detection for several different biomarkers in a single assay by using different QDs as acceptors and Tb as a donor, respectively. Qiu *et al.*[324] first developed duplexed Tb-to-QD FRET immunosensors to simultaneously determine EGFR and HER2 biomarkers in solution and confirmed that the usage of small antibody fragments as an alternative to intact antibody could improve the sensitivity (Figure 10B). This group also demonstrated a time-gated fluorescent FRET nanosensor using a Tb-to-QD pair for simultaneous detection of multiplexed miRNA in solution based on different commercial QDs in combination with hybridization assays (Figure 10C).[325] Beyond single-step donor−acceptor FRET pairs, incorporating QDs within multistep FRET relays has recently gained traction for multiplexed sensing. Algar and co-workers[326] reported a time-gated two-step FRET relay using single-colored QD as acceptors and donors at the same time. By coupling with Tb complex or Alexa Fluor 647-labeled peptide or oligonucleotide-mediated co-assembly, the FRET relay was demonstrated in the fabrication of a no-wash multiplexing biosensor for protease activity monitoring and DNA hybridization assay. During the same year, the author also reported a two-step time-gated FRET relay for multiplexed tracking of the enzymatic activity of trypsin, chymotrypsin, and pro-chymotrypsin through ratiometric and kinetic measurements.[327] Later, another interesting three-step FRET relay consisting of a central QD surrounded by multiple Alexa Fluor 555 and Alexa Fluor 647 dyes was designed. Energy transfer occurred from the QD donor to the Alexa Fluor 555 ($FRET_1$), then to the Alexa Fluor 647 ($FRET_2$), and, to a lesser extent, directly from the QD donor to the Alexa Fluor 647 ($FRET_3$) (Figure 10D).[328] The multistep FRET relay was successfully applied in duplex sensing of trypsin and chymotrypsin.

UCP-Based FRET Biosensors. Lanthanide-doped UCPs have attracted considerable interest because of their distinct advantages, including long luminescence lifetimes, large anti-Stokes shifts, narrow emission bands, high resistance to photobleaching, and low toxicity.[329,330] More importantly, the UCPs provide an outstanding advantage stemming from their distinct NIR excitation for UCPs that can cause no or weak background fluorescence, thereby contributing to increasing signal-to-noise ratio and high detection sensitivity when compared with other luminescent nanomaterials.[331] These properties make UCPs as promising background-free luminescent probes for diverse biological applications, like biosensing and bioimaging.[332,333] Particularly, using UCPs as donors for no-wash energy-transfer assays (UC-FRET) have been extensively developed in the past decade.[334,335] Here, a comprehensive list of advances in the field of *in vitro* no-wash detection will be provided with special emphasis on early detection of cancer biomarkers. The first application of UCPs as energy donors in UC-FRET biosensors for avidin dates back to the work by Wang *et al.*[336] Soon afterward, various types of UC-FRET biosensors were explored and used for *in vitro* detection of nucleic acids, proteins, enzymes, as well as small organic molecules.[332,334] For DNA detection, a typical design strategy mainly involves sandwich hybridization, where both UCP-labeled DNA and dye-conjugated DNA can hybridize with complementary targets acting as UC-FRET donors and acceptors, respectively (Figure 11A). Using this sandwich hybridization format, several groups have developed UC-FRET systems to detect different target DNA molecules.[337,338] Combined with two different dye acceptors, a duplex no-wash sandwich hybridization UC-FRET sensor was developed to measure two different target DNAs.[339] Another simpler hybridization-based approach for designing no-wash UC-FRET biosensor was explored by using the UCPs and an intercalating dye as energy donor and acceptor, respectively. In this strategy, the intercalating dye acceptor would exhibit higher fluorescence due to its intercalation with dsDNA through target DNA hybridization with the probe. Based on this mechanism, Jiang *et al.*[340] successfully detected RNA with UCPs and BOBO-3 as donor and acceptor intercalating dyes, respectively (Figure 11B). Another two similar studies from Kumar *et al.*[341] and Guo *et al.*[342] were designed for the detection of DNA through intercalating fluorescence dye molecules of SYBR Green I and POPO-3, respectively. Recently, Wang and colleagues[343] reported a UC-FRET biosensing strategy for DNA assay, which is similar to GO-based fluorescence quenching sensors (Figure 11C). To achieve the UC-FRET pair, the self-assembly of the UCP donor and the poly-*m*-phenylenediamine (PMPD) nanosphere acceptor was obtained based on the $\pi-\pi$ stacking interaction between ssDNA and the π-rich electronic structure of PMPD. After the formation of dsDNA between ssDNA and target DNA, the PMPD energy acceptor was detached from the UCP donor surface owing to the weak interaction between dsDNA and PMPD. Another effective method to fabricate UC-FRET biosensors is through the combination of UCPs as donors and dye-labeled MBs as acceptors. The addition of target DNA could induce the separation of UCP donors and acceptor molecules, thus producing a change in FRET efficiency. Recently, several researchers have successfully used this strategy in target DNA determination.[344,345] More recently, Ding *et al.*[346] demonstrated a ratiometric fluorescence UC-FRET nanoprobe with the conjugation of specific MBs containing a quencher (BHQ-1) and UCPs for visual detection of thymidine kinase 1 mRNA (Figure 11D). The target mRNA binds with the MBs and triggers the release of the chemotherapy drug (DOX) to deliver the drug in MCF-7 and A549 tumor cells. Apart from nucleic acid detection, UCP-based no-wash UC-FRET assays also exhibit an excellent application in tumor-related enzyme detection.[347] Wang *et al.*[348] presented a UC-FRET sensor for ultrasensitive detection of hyaluronidase by using UCPs and PMPD as energy-transfer donor and acceptor with the combination of enzyme-catalyzed hyaluronic acid cleavage that could induce the separation of donor and acceptor, thereby favoring an efficient FRET process (Figure 11E). Moreover, Liu *et al.*[349] used tetramethylrhodamine (TAMRA)-labeled peptide substrate as an UC-FRET

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acceptor for monitoring protein kinase activity, where the existence of target enzyme facilitates TAMRA-labeled peptide phosphorylation and the phosphorylated TAMRA-labeled peptides specifically captured on the UCPs, thus generating a low-background UC-FRET sensing platform. Finally, to facilitate early detection of protein biomarkers, Li *et al.*[350] recently reported a no-wash FRET sensor for ultrasensitive detection of CEA by using palladium nanoparticles as a fluorescence quencher of UCPs in combination with aptamer-induced conformational change (Figure 11F).

NANOPARTICLES AS ENHANCERS

As mentioned above, fluorescence-based analytical technology has been extensively used in bioimaging, medical diagnostics, as well as biosensing. However, relatively low quantum yields of fluorescence molecules lead to poor detection sensitivity, limiting their further practical applications. Metal-enhanced fluorescence (MEF), which occurs between the intense plasmon-induced electric field and the excited-state of fluorophores located in close proximity to noble metal nanostructures, such as the AuNPs and AgNPs, is used to improve the sensitivity of fluorescence measurements. Although many literatures on biosensor applications of MEF involve nanomaterial textured surfaces, only few researchers have developed MEF-based fluorescence biosensors for tumor biomarkers in solution. Li *et al.*[351] described a solution-based MEF platform for target DNA detection by applying a DNA oligonucleotide bridge to form a QD-AuNP enhancement system, in which the distance of QDs and AuNPs could be easily controlled by changing the base numbers of oligonucleotide. Cho and co-workers[352] reported on a AuNP-based MEF aptasensor for $VEGF_{165}$ based on target binding-induced aptamer conformational changes that can result in the detachment of unfolded Cy3-aptamer conjugates, thereby causing the inactivation of MEF for target detection. Additionally, several studies have focused on AgNP-enhanced FRET sensors for no-wash detection of cancer protein biomarkers.[353−355] In these sensing systems, AgNPs were used as a framework to construct MEF-FRET double models, where the presence of target would trigger the dissociation of the MEF-FRET structure, thus disrupting the FRET response. More recently, Li *et al.*[356] displayed a similar AgNP-enhanced FRET nanosensor for quantitative detection of PDGF in solution through coupling AgNP-based MEF with two FRET modes. Moreover, based on this strategy, AgNP-enhanced FRET sensor can also directly detect human acute lymphoblastic leukemia CCRF-CEM (CCL-119, T-cell line) in solution.[357]

BIOLUMINESCENE RESONANCE ENERGY TRANSFER BIOSENSORS

Although fluorescence-based techniques are very popular in the field of no-wash detection, to some extent, photobleaching and autofluorescence limit the usefulness of fluorescence.[358] In addition, an external light source is required to excite the fluorescence materials, thus the fluorescence-based assay may be impractical in tissues that are easily damaged by the excitation light or that are photoresponsive (*e.g.*, retina). To overcome these problems, bioluminescene resonance energy transfer (BRET) sensor was brought up as a complementary method to fluorescence-based detection. Compared with FRET, BRET depends upon a nonradiative energy transfer between a donor of a bioluminescent enzyme and an acceptor of a fluorescent molecule on the basis of spectral overlapping. BRET, as a naturally occurring phenomenon, provides potential advantages over FRET, such as an increased signal-to-noise ratio, decreased background interference, reduced risk of photobleaching, as well as elimination of the need for external excitation.[359] Early BRET systems were mainly employed for investigating the interaction of protein−protein or protein−ligand species through *Renilla* luciferase (Rluc) or related mutants with an increased light yield as the energy donor and variants of green fluorescent protein (GFP) with suitable spectral overlapping properties as the energy acceptor. Recently, BRET-based biosensing platforms have been widely explored as a tool for no-wash detection of tumor-related biomarkers, including RNA,[360] DNA,[361] VEGF,[362] thrombin,[363] caspase-3,[364] and Src tyrosine kinase.[365] Nonetheless, the sensitivity of these assays could still be improved. To enhance the detection sensitivity and broaden the applicability of BRET-based sensors, several attempts have been devoted to constructing a kind of BRET nanosensor by employing other energy acceptors as an alternative for the commonly used GFP, such as organic fluorophores, AuNPs, QDs, and so on. For example, Kumar *et al.*[366] and Cissell *et al.*[367] fabricated two single-step BRET biosensors to determine target DNA based on sandwich-type and competitive hybridization, respectively. In the two assays, the QDs with high luminescence were used as a BRET energy acceptor of a Rluc donor. Later, a QD-based BRET biosensor was designed by Yao and co-workers[368] for the detection of MMP-2 *via* enzyme-catalyzed hydrolysis of peptide substrates that can disrupt BRET efficiency. Moreover, AuNPs can be applied to design nanosensors as energy acceptors for Rluc donors owing to its superquenching ability. For instance, Kim *et al.*[369] presented a AuNP-based no-wash BRET sensor for monitoring the activity of MMP-2 based on the digestion of substrate peptide that causes the release of Rluc from the AuNP surface, thereby generating an effective BRET change. A similar AuNP-based BRET sensor was reported by Chen *et al.*[370] for the detection of thrombin in solution.

OUTLOOK OF FLUORESCENT NO-WASH BIOSENSORS

Although great progress has been achieved in fluorescence-based no-wash biosensors, there still remains great challenges that hamper their routine practice in clinical diagnosis. For MNC-based fluorescence biosensors, the size-controlled synthesis methods of MNCs with high purity and high quality are still lacking, and almost all currently used MNCs suffer a common problem of relatively low quantum yield below 10%. Moreover, the fundamental understanding of the structure and the underlying luminescent mechanisms play a pivotal role in basic research and practical applications. For energy-transfer-based fluorescent biosensors, the major challenge lies in the precise control of the distances between the energy donors and the energy acceptors, which greatly affect the energy-transfer efficiency and the analytical reproducibility. Additionally, like other nanoparticle-based sensors, the stability of nanomaterials, the nonspecific adsorption from proteins and other biomolecules on the nanomaterial surface, and mass-scale production still remain a major task, which will determine their practical applications. Furthermore, eliminating the interference of the autofluorescence background signals from biomolecules in real samples is vital to their clinical use like other fluorescent approaches. Besides, the fluorescence is vulnerable to environmental changes such as pH and ion strength, which can easily

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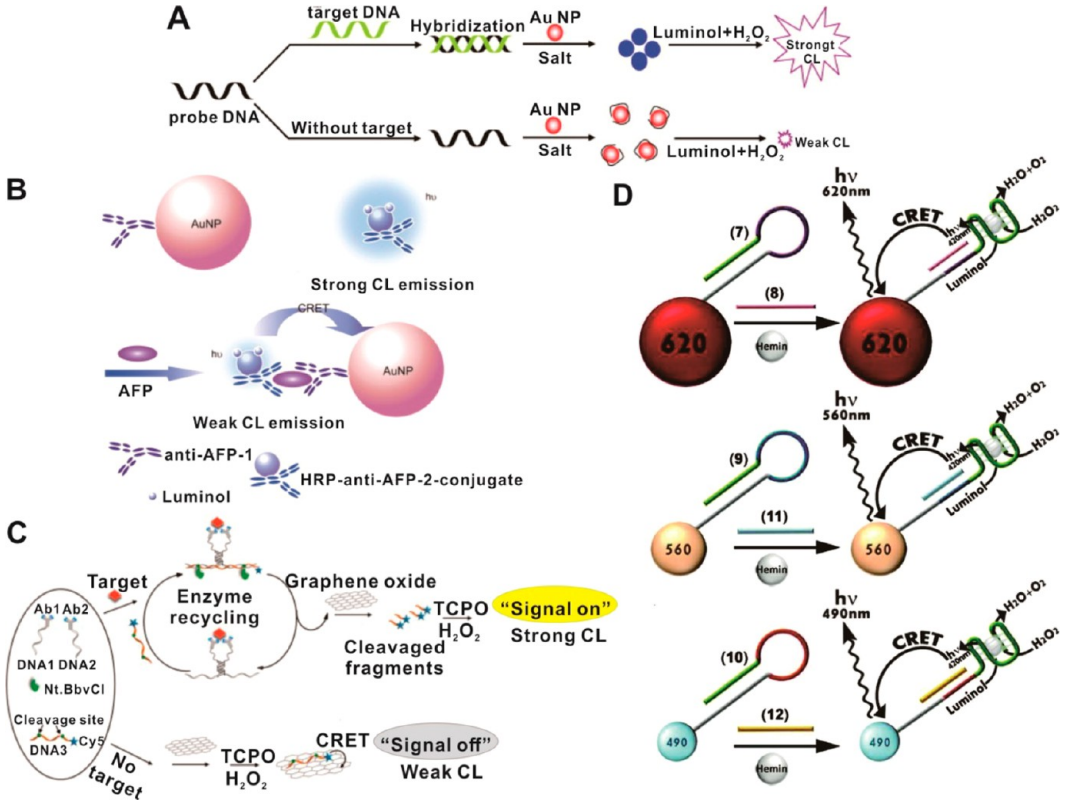

Figure 12. (A) Label-free CL detection of DNA hybridization using GNPs probes. Reprinted with permission from ref 382. Copyright 2009 Elsevier B.V. (B) CRET-based immunoassay for AFP. Reprinted with permission from ref 398. Copyright 2010 Elsevier B.V. (C) Proximity hybridization-regulated CRET for homogeneous detection of CEA. Reprinted with permission from ref 406. Copyright 2016 Elsevier B.V. (D) CRET sensing platform for multiplexed analysis of the different target DNAs using three different sized CdSe/ZnS QDs emitting at 620, 560, and 490 nm. Reprinted with permission from ref 411. Copyright 2011 American Chemical Society.

cause the fluorescence signal to fluctuate, thus resulting in inaccurate readouts. Lastly, although fluorescence-based assays exhibit great potential in multiplexed sensing, and some progress has been attained, the emission spectra from different luminescent materials are to some extent overlapping, which lead to the inaccuracy of detection signals. In brief, further study should be devoted to improving the MNC synthesis to raise quantum yield, precisely controlling the distance of donor and acceptor, increasing energy-transfer efficiency, as well as developing fluorescent analytical methods and nanomaterials with excellent properties to effectively overcome these problems which limit their clinical utility.

CHEMILUMINESCENT NO-WASH BIOSENSORS

Chemiluminescence (CL) is the generation of electromagnetic radiation in the visible or near-infrared region. CL phenomena can take place in aqueous phases or solid phases and are generated by redox reactions between at least two CL reagents under proper reaction conditions. During the CL reaction process, the produced electronically excited intermediate is unstable and relaxes to the ground state with light emission. In general, the CL method is established by direct oxidation of target compound to produce emitting species or by indirect enhancing or inhibitory effects of certain luminescent compounds. As a powerful analytical technique, the CL method exhibits high sensitivity, good specificity, rapid analysis, simple operation, wide linear range, and little background interference.[371,372] Compared with fluorescence-based detection, CL provides at least 1 or 2 orders of magnitude improvement in sensitivity and a broader dynamic range.[373] Although CL methods based on solid formats have gained a wide range of applications in the past few decades, such assay formats are heterogeneous with washing steps, which is not only laborious and time-consuming but also makes the analytical performance more complex and susceptible to cumulative error. As such, CL occurring in solution has been explored in the recent years as a main analytical platform for sensitive detection of various target analytes in clinical diagnosis because of its simplicity, rapidity, and wash-free method.[374,375] A large number of no-wash CL biosensors with excellent analytical performances have emerged through applying nanomaterials as luminescence reagents, label carriers, catalysts, reductants, as well as energy acceptors of CL reactions.

CHEMILUMINESCENCE BIOSENSORS

CL biosensors have been developed to analyze a variety of biomolecules due to their high sensitivity and have been widely implemented in clinical, environmental, and biochemical analyses. Most of the well-established CL assay methods use enzymes as CL labels. Currently, one of the most sensitive and widely used CL systems is based on the CL reaction of luminol–H_2O_2–horseradish peroxidase (HRP). In developing solution-based CL biosensors, the DNAzymes and hemin/G-quadruplex nanostructures generated by the introduction of target have been used as an amplifying label for the analysis of target DNA and aptamer complexes because they show HRP mimicking activities that can catalyze the CL reaction of luminol–H_2O_2. Based on this strategy, a series of no-wash CL

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biosensors have been fabricated for SNP genotyping[376] and target DNA detection.[377,378] Thereafter, some signal amplification strategies were introduced to amplify the CL detection signal using nanomaterials. Zhou *et al.*[379] used AuNPs as a carrier to assemble two DNAzyme halves through hybridization with corresponding linking DNA sequences that can effectively recognize target DNA. With target DNA, the DNAzyme halves release from the AuNP surface into the solution owing to the competitive recognition of target DNA. The free DNAzyme halves then combine with hemin to form a hemin/G-quadruplex structure, thereby producing an amplified CL signal. Gao *et al.*[380] employed single-walled CNT to amplify the CL signal by eliminating high background signal for ultrasensitive analysis of target DNA in solution. Luo *et al.*[381] first demonstrated the excellent inhibiting capacity of GO toward the HRP-mimicking DNAzyme activity. Based on this, a no-wash CL biosensing platform was explored to detect DNA. Although the usage of enzymes as labels can largely amplify the CL detection signal, enzyme labels suffer several inherent disadvantages of easily denaturalized characteristics, short lifetime, and low stability. To address these problems, using nanomaterials as alternative enzyme labels for CL signal amplification in the liquid phase has received much attention due to their higher specific activities, stabilities, as well as sensitivities. Qi *et al.*[382] first demonstrated the catalytic activity of AuNPs on the CL reaction of luminol-H_2O_2 and found that the catalytic activity was related to the colloidal state of AuNP in solution. The aggregated AuNPs showed a higher catalytic activity than the dispersed AuNPs (Figure 12A). Using target DNA hybridization, the author constructed a no-wash CL sensor, in which the ssDNA could stabilize the dispersed AuNPs, even in high-salt concentration compared with the dsDNA. Subsequently, the author reported a similar CL sensor for thrombin detection using aptamer-mediated AuNP aggregation or dispersion.[383] Combined with traditional immunoreaction recognition-induced cross-linking aggregation, a no-wash CL biosensor using AuNPs as catalysts was proposed for protein biomarker detection.[384,385] No-wash CL immunosensor is an excellent analytical technique of CL combined with conventional immunoassay, which has been extensively applied for the analysis of protein markers. The first demonstration of no-wash CL immunosensor was offered by Tatsu and co-workers.[386] In following, Akhavan-Tafti *et al.*[387] reported a no-wash CL immunosensor for PSA detection, where the formation of target-mediated sandwich immunocomplexes resulted in the close proximity of acridan-based chemiluminescent compounds and HRP enzyme, thus producing a CL signal upon addition of a trigger solution. To lower the detection limit, an amplified CL immunosensor for the detection of CEA[388] and carbohydrate antigen 19-9 (CA 19-9)[389] was designed by applying AuNPs as HRP and HRP-mimicking DNAzyme carriers, respectively.

CHEMILUMINESCENCE RESONANCE ENERGY-TRANSFER BIOSENSORS

Analogous to FRET and BRET, CRET involves a nonradiative energy transfer from a chemiluminescent donor to an acceptor molecule. The CRET takes place based on the oxidation of a chemiluminescent substrate without an excitation light source, which can minimize nonspecific signals and avoid fluorescence bleaching, giving CRET an advantage in sensitivity in tumor-related nucleic acid detection, protein detection, and enzyme activity monitoring.[390,391] Conventional CRET-based biosensors apply organic dye as CRET acceptors for enzyme-mediated CL reactions to achieve target detection. Emulating this method, various targets such as thrombin[392,393] and miRNA-122[394] were successfully detected. To improve the efficiency of energy transfer, more nanomaterials have been explored as an alternative to organic dyes for CRET acceptors, which provide benefit through higher sensitivity in biomolecule detection. Because of the superquenching ability of AuNPs to CL, Xu and colleagues reported a AuNP quenching-based CRET sensor for no-wash DNA detection on the basis of preferential absorption of acridinium ester-labeled ssDNA of unmodified AuNPs over dsDNA.[395] Later, a sensitive AuNP quenching-based CRET aptasensor was designed for target thrombin detection in solution.[396,397] Recently, a no-wash CRET immunosensor for AFP analysis was published by Huang *et al.*,[398] who used AuNPs to quench the CL reaction of luminol-H_2O_2 with the help of specific immunological recognition motifs (Figure 12B). In addition, AuNP quenching-based CRET sensors for monitoring tumor-related enzyme activities in solution have a great clinical relevance. Huang *et al.*[399] developed a CRET biosensor for caspase 3 activity analysis based on enzyme-catalyzed cleavage of HRP-labeled peptide substrates attached on AuNP surface, thus disrupting CRET. Similarly, a CRET sensor platform was offered for sensitive analysis of trypsin activity by using BSA-stabilized AuNCs as a CL energy acceptor. GO is another superquencher for CL.[400] Owing to its stronger binding ability with ssDNA *via* $\pi-\pi$ stacking interactions over dsDNA, GO quenching-based CRET sensor was fabricated for target DNA detection in solution.[401,402] Afterward, an improved no-wash CRET sensor for ultrasensitive DNA detection was constructed by the integration of exonuclease III-assisted target recycling amplification.[403] Moreover, GO quenching-based CRET sensor was also applied for no-wash detection of protein markers. Zhou *et al.*[404] designed a strategy for CEA detection by target-mediated dissociation of HRP-tagged CEA aptamer to disturb the CRET of the HRP-luminol-H_2O_2 CL system and GO quencher. Immunoassay acts as one of the most important analytical methods in protein analysis. Through the combination of CRET assay, a no-wash CRET immunosensor was recently displayed for the detection of C-reactive protein in solution by Lee and co-workers,[405] who used GO as energy acceptor to quench the CL of HRP-luminol-H_2O_2. Thereafter, a similar no-wash GO-quenching CRET immunosensor for quantitative analysis of CEA was presented through the combination of immunoreaction-regulated close proximity of Cy5 donor and GO acceptor (Figure 12C).[406] Moreover, combined with exonuclease III-assisted target recycling amplification, an improved CRET sensing can be employed to sensitively measure DNA methyltransferase activity by using GO as CL quencher.[407] Meanwhile, the proposed biosensing method also exhibited an excellent sensitivity for site-specific determination of DNA methylation. Besides the forementioned nanoquenchers to CL, other nanomaterials, including CNPs,[408] WS_2 nanosheet,[409] as well as polydopamine nanospheres,[410] have been integrated as excellent quenchers to CL for establishing highly sensitive CRET nanosensors for different biomolecules. The usage of QD as energy acceptors of the hemin/G-quadruplex-luminol-H_2O_2 CL system in solution was introduced to design no-wash CRET sensors for sensitive detection of target analytes. Based on this concept, Willner's group has produced a series of outstanding works on DNA and protein detection. In 2011, this group reported on a CRET-based sensor to analyze target

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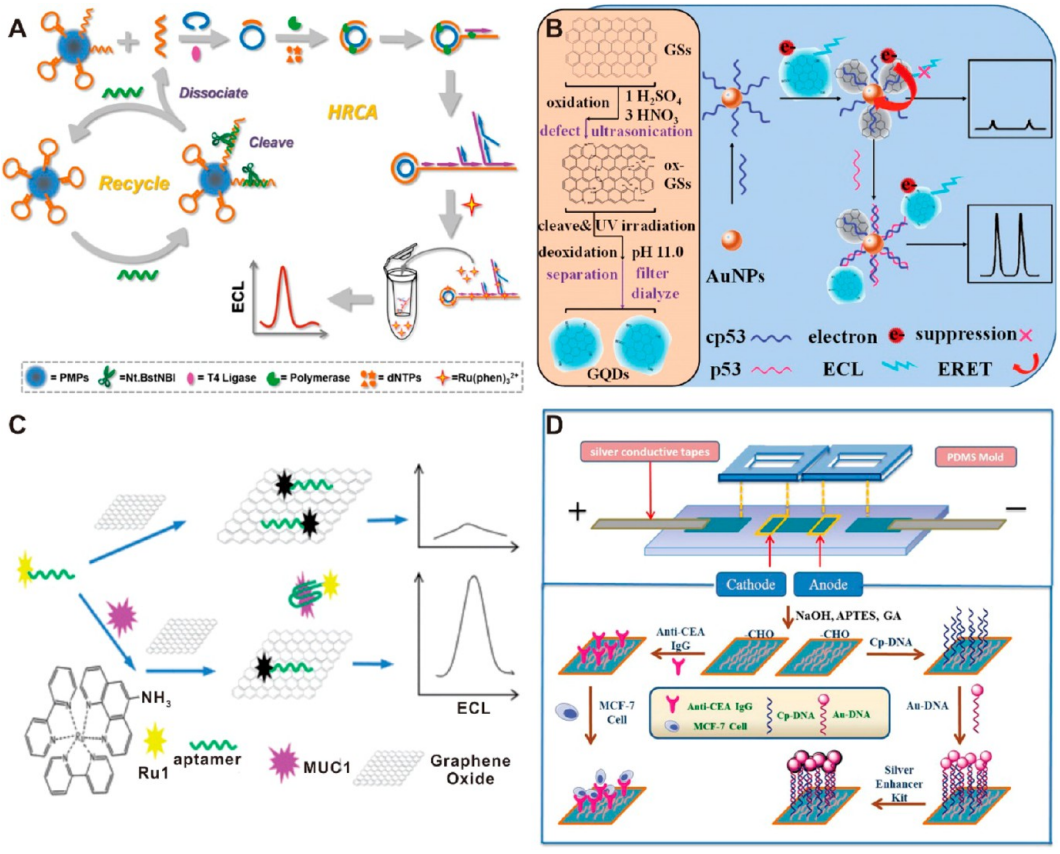

Figure 13. (A) Cascade signal amplification-based ECL biosensor for p53 DNA. Reprinted with permission from ref 414. Copyright 2016 American Chemical Society. (B) AuNP-induced ECL quenching of the GQDs for target p53 DNA. Reprinted with permission from ref 418. Copyright 2014 The Royal Society of Chemistry. (C) GO-induced ECL quenching for Mucin 1 protein and MCF-7 cancer cells. Reprinted with permission from ref 421. Copyright 2012 The Royal Society of Chemistry. (D) Time-resolution ECL biosensor for MCF-7 and A549 cancer cells based on closed bipolar electrode. Reprinted with permission from ref 425. Copyright 2016 American Chemical Society.

DNA, where nucleic acid hairpin structure-capped CdSe/ZnS QDs were opened by target DNA to expose the HRP-mimicking DNAzyme sequence for the formation of a hemin/G-quadruplex DNAzyme, thereby favoring an effective CRET (Figure 12D).[411] Additionally, the author found that CRET-based biosensors show potential for multiplex analyses for simultaneous detection of multiplexed target DNA with different sized QDs as CL energy acceptors in one single assay. Later, several similar QD-based CRET nanosensors were developed for the determination of cancer protein markers, like thrombin[412] and VEGF,[301] respectively.

ELECTROGENERATED CHEMILUMINESCENCE BIOSENSORS

Electrogenerated chemiluminescence or electrochemiluminescence (ECL) is a special kind of CL triggered by an electrochemical reaction rather than a chemical reaction. As a marriage of CL and electrochemistry, ECL not only has the advantages of CL detection but also some specific advantages such as an enhanced selectivity, extended analytical application, and improved detection spatial resolution. These superiorities make ECL technology a popular method for applications in biological analysis. However, the commonly used ECL sensors are generally heterogeneous and conducted on an electrode surface, which often requires repeated incubation, washing, and separation steps. In contrast, no-wash ECL biosensors do not require washing steps and have become a promising method for early detection of cancer biomarkers *in vitro* with simplicity and automation. Lou *et al.*[413] first developed an ECL sensor for highly sensitive DNA detection in solution through graphene QDs (GQDs) as ECL signal labels in combination with endonuclease cleavage and bidentate chelation. Hereafter, Yang and co-workers[414] designed a no-wash ECL biosensor for sensitive detection of p53 DNA on the basis of dual signal amplification of nicking endonuclease-assisted target recycling and magnespheres paramagnetic nanoparticle-hyperbranched rolling circles, where a large number of dsDNAs accumulated from target-triggered dual signal amplification were introduced for the insertion of the $Ru(phen)_3^{2+}$, as a ECL signal reporter (Figure 13A). Analogous to DNA analysis, no-wash ECL biosensing for sensitive miRNA assay has also been demonstrated. Through the integration of T7 exonuclease-assisted cyclic amplification and 3D DNA-mediated silver enhancement, Zhang *et al.* presented an ECL biosensor for no-wash detection of miRNA using GQDs as ECL signal probes.[415] In this assay, the helper DNA first hybridized with hairpin probe coated on the electrode surface to expose the stem that can hybridize with target miRNA to form a DNA/RNA duplex. Then the DNA/RNA duplex was digested with the T7 exonuclease to release target miRNA for triggering of the next recycling phase. To further improve the detection signal, 3D AgNP-based DNA networks were proposed with the hydroquinone-induced reduction of Ag^+. Moreover, no-wash ECL biosensors have significant implications for protein and

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enzyme analysis. For instance, Jie *et al.*[416] designed an ECL aptasensor for highly sensitive assay of thrombin in solution based on QDs as an ECL label and target-triggered enzyme-mediated multiple DNA cycle amplification strategy. Luo and colleagues[417] illustrated an ECL method for determining DNA methyltransferase activity in solution through using tris(2,20-bipyridine)ruthenium-AuNP composite as an ECL signal probe and ferrocene as a quencher. The quenched ECL signal induced by DNA hybridization was recovered in the presence of DNA methyltransferase.

With the progress of ECL analysis, ECL resonance energy transfer (ECL-RET) has received increasing attention in sensing applications of protein, DNA, and even cells. When the electrochemical donor transfers energy to ECL acceptors under a potential, the signal intensity of ECL is greatly enhanced. Thus, the sensitivity of ECL-RET-based sensors is much higher than that of normal ECL sensors, making the no-wash ECL-RET-based sensors a robust analytical platform in the field of cancer biomarker detection *in vitro*. Lu *et al.*[418] used AuNPs as quenching acceptors for GQD ECL donors to establish an ECL-RET-based sensor for DNA damage detection in solution, in which the quenched ECL signal of the GQDs was realized on the basis of the noncovalent binding of the AuNP−ssDNA complex to the GQDs, allowing the AuNPs−ssDNA to fall in close proximity to the GQDs (Figure 13B). The target p53 DNA can hybridize with AuNPs-ssDNA adsorbed on the GQD surface to form AuNPs-dsDNA, weakening the interaction of AuNPs and the GQDs that cause detachment of AuNPs. Thus, the ECL signal of the GQDs is recovered. Zhang and colleagues[419] described a DNA nanomachine-based ECL-RET sensing platform for miRNA-21 from human breast cancer MCF-7 cells by employing Alexa Flour 488 as the donor and CdSe@ZnS QDs as the acceptor, where a dual amplification strategy of target recycling and signal transformation was applied to accumulate universal DNA reporters that can cause the conversion of a DNA tweezer state from off to on through the hybridization of both strands. The close proximity of QDs and Alexa Flour 488 on the two arms further increases ECL intensity of QDs owing to RET. Moreover, no-wash ECL-RET-based biosensors have also been involved the detection of protein and enzyme markers. Coupled with AuNR-induced quenching of near-infrared ECL signal of QDs, a no-wash ECL-RET biosensing for thrombin was achieved by using target-mediated aptamer structural change that results in the dissociation of AnNRs from QD, further disrupting the ECL-RET efficiency.[420] GO was also designed as an ECL-RET energy acceptor to quench the ECL signal. Wei *et al.*[421] constructed an ECL-RET sensing strategy for protein analysis in solution by employing bis(2,20-bipyridine)-(5-aminophenanthroline)ruthenium(II) (Ru1) as an ECL donor and GO as an acceptor (Figure 13C). The aptamer was used to recognize the target MUC1 protein and as a carrier to conjugate the ECL label of Ru1. The specific interaction of aptamer and target can induce the detachment of the Ru1-aptamer from the GO surface and give rise to the restoration of ECL signal. Moreover, the developed method can also sensitively detect the presence of MCF-7 cancer cells. Through applying GQD as an ECL donor and GO as an acceptor to fabricate an ECL-RET pair, Liang and co-workers[422] successfully monitored protein kinase activity in solution. Dong *et al.*[423] demonstrated a no-wash detection method for thrombin determination based on the ECL-RET of QD energy as an acceptor and luminol ECL as a donor with target-induced aptamer conformation transformation. Furthermore, the no-wash ECL-RET sensing can be directly employed to measure the presence of tumor cells. Wu *et al.*[424] first used CdS QDs and $Ru(bpy)_3^{2+}$ as ECL-RET energy donor and acceptor, respectively, to build a no-wash bionsensor for SMMC-7721 cancer cell assay. Recently, an interesting ECL-RET biosensor was reported for the ultrasensitive detection of MCF-7 cancer cells in solution, where luminol ECL was a donor and Ag shell−Au core was a quenching acceptor (Figure 13D).[425] Under a certain potential, the Ag from the Ag shell−Au core was dissolved, and the quenched ECL signal was recovered. Thus, the amount of MCF-7 cancer cells can be accurately quantified by recording the ECL recovery time rather than ECL intensity.

LUMINESCENT OXYGEN CHANNELING IMMUNOASSAY

As previously mentioned, energy-transfer-based no-wash detectors have been widely reported in the literature. However, we found that these assays exhibited better analytical performance in measuring nucleic acid markers rather than protein biomarkers. The possible reason for this observance is that the target proteins and antibodies (6−10 nm) exhibit a larger size than other smaller molecules, like DNA. After the formation of sandwich immunocomplexes of donor-labeled antibody−protein−reporter antibody-conjugated acceptor, the distance between the donor and acceptor exceeds the optimal distance for energy transfer with high efficiency, which would deteriorate the detection sensitivity of energy-transfer-based assay. Although some strategies, such as using small antibody fragments or nanobodies as an alternative to conventional antibody, have been proposed for improving the energy-transfer efficiency of donor and acceptor in no-wash immunosensors of protein, there is still room for improvement. In the past several decades, a luminescent oxygen channeling immunoassay was designed for biomarker discovery. Later, PerkinElmer further developed this method as an amplified luminescent proximity homogeneous assay (AlphaLISA). AlphaLISA includes two primary assay formats, sandwich and competition, which can be applied to analyze proteins and small molecules, respectively. In a typical sandwich AlphaLISA for protein, the sandwich immunocomplex is obtained by the specific immunological recognition of target protein, a biotinylated antibody, and a secondary antibody conjugated to AlphaLISA acceptor beads.[426] The streptavidin-coated donor beads are added to bring donor and acceptor beads into close proximity with the biotin−streptavidin system. The excitation of the donor beads by laser irradiation at 680 nm produces a flow of singlet oxygen species that trigger a cascade of chemical events in the nearby acceptor beads, which results in a chemiluminescent emission at 615 nm (Figure 14). As a class of special CL assay, AlphaLISA assays are based on the simple "mix-and-measure" protocol, which effectively avoids multiple washing steps, decreases the total assay time, and increases the dynamic range compared to ELISA. Due to these outstanding advantages, the AlphaLISA immunoassay platform has facilitated a wide range of applications in the analysis of cancer-related protein biomarkers over recent years, including fucosylated glycoproteins,[427] core-fucosylated E-cadherin,[428] cytokeratin 19 fragment antigen,[429] Na^+/H^+ exchanger regulatory factor 1,[430] and human epididymis protein 4.[431] Nevertheless, the current AlphaLISA system lacks the capacity for multiplexed detection. To overcome this problem, an

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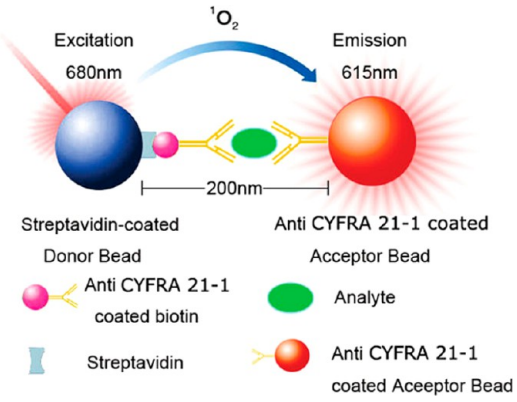

Figure 14. Schematic representation of AlphaLISA technology for CYFRA21-1. Reprinted with permission from ref 429. Copyright 2013 Wiley Periodicals, Inc.

aqueous two-phase system composed of the phase-separating polymers, polyethylene glycol, and dextran was put forward by Simon and colleagues[432] to enable multiplexing AlphaLISA for simultaneous analysis of multiple protein biomarkers. Here, the aqueous two-phase system effectively separates antibody/bead reagents stably in the dextran phase, thereby avoiding the cross-reactions of mismatched antibody reagents, and allows discrete readouts from multiple CL signals patterned within one well.

OUTLOOK OF CHEMILUMINESCENT NO-WASH BIOSENSORS

Over the past few decades, a lot of research effort has been invested in the fabrication of chemiluminescent no-wash biosensors with better sensitivity, better selectivity, reliability, ease of operation, and low cost. However, there are still many problems to be remedied. The chemiluminescent biosensors suffer from relatively poor selectivity and can respond nonspecifically to a series of compounds rather than a single one. In addition, CL intensity is very sensitive to various environmental factor changes, which can produce big differences in emission intensity in different systems. Furthermore, the matrix interference from complex samples can significantly disturb the detection stability and accuracy. Moreover, chemiluminescent biosensors still require large, expensive, and complicated instruments to complete the acquisition of signal. Besides, like other nanomaterial-based analytical approaches, the stability, surface modification, and nonspecific adsorption is also worth noting. Finally, although AlphaLISA has been commercially used and has gained wide application, the major challenge is the lack of multiplexed detection ability. Thus, future research trends may include the following aspects, such as understanding the underlying mechanism of chemiluminescent reaction, increasing the selectivity to target analytes, enhancing the robustness and reproductivity, designing smaller and more portable instruments, developing more nanomaterials with different sizes and shapes to fabricate excellent chemiluminescent systems, as well as exploring multiplexed detection capabilities without any confounding interference.

ELECTROCHEMICAL NO-WASH BIOSENSORS

Electrochemical sensing combines sensitive electrochemical transducers and specific biological recognition processes. Electrochemical devices are generally made of a biological recognition element that can selectively bind with the target analyte and produce an electrical signal that is relevant to the concentration of the target analytes in the sample solution. Owing to the high sensitivity of electrochemical transducers, electrochemical detection technologies have proven to be a powerful sensing strategy superior to various traditional analytical methods.[433] Not only that, but electrochemical biosensors also provide other advantages, including rapidity, easy operation, low cost, less power requirement, and the potential for point-of-care testing. Moreover, electrochemical biosensors exhibit great promise for the development of microscale analytical platforms since they are very easily miniaturized and integrated without compromising their analytical performances. In the last few decades, a growing trend in electrochemical biosensors has been observed in the field of bioanalysis. However, most of these electrochemical biosensors are designed on the electrode surface with heterogeneous formats, which often involve multiple separation and rinsing steps. Moreover, the heterogeneous reaction models largely decrease the repeatability and reproducibility.

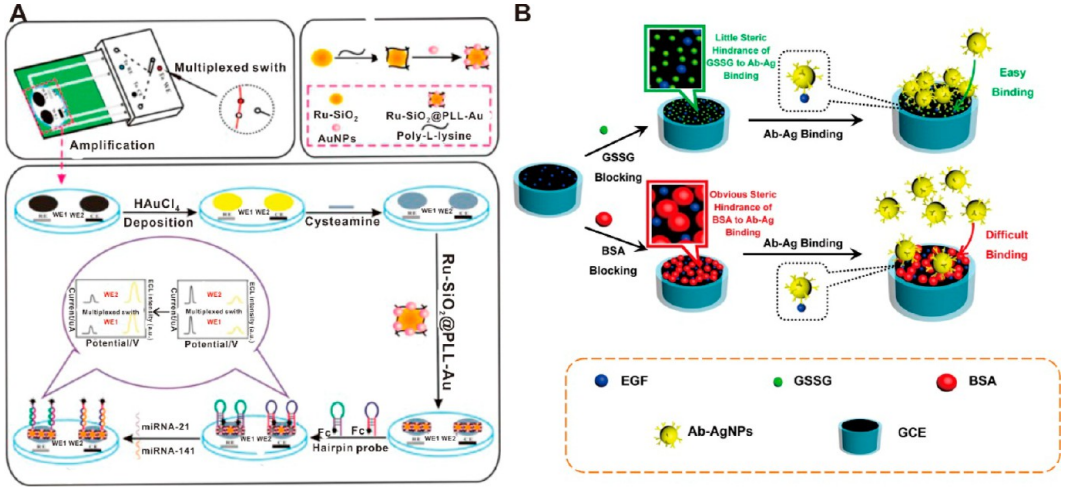

Figure 15. (A) ECL ratiometric biosensor array for multiplexed detection of miRNA-21 and miRNA-141. Reprinted with permission from ref 439. Copyright 2015 Elsevier B.V. (B) Electrochemical immunosensor for epidermal growth factor using GSSG and BSA as blocking reagent. Reprinted with permission from ref 442. Copyright 2015 American Chemical Society.

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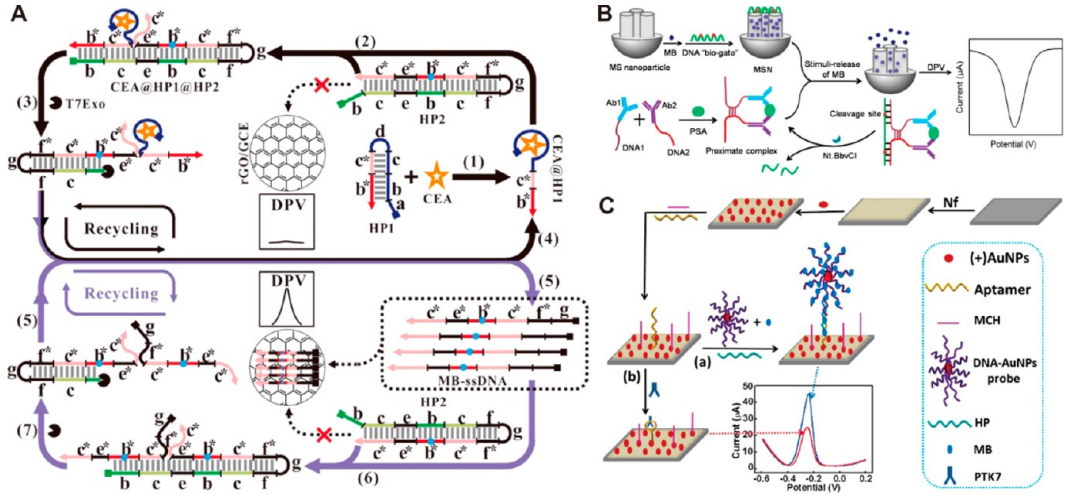

Figure 16. (A) GO-based no-wash ECL aptasensor for CEA with T7 exoassisted target-analog recycling circuit with isothermal signal amplification. Reprinted with permission from ref 450. Copyright 2016 American Chemical Society. (B) No-wash ECL immunosensor using proximity hybridization-responsive mesoporous silica nanoprobe. Reprinted with permission from ref 452. Copyright 2014 American Chemical Society. (C) No-wash ECL strategy for protein tyrosine kinase-7 detection based on the signal change of methylene blue. Reprinted with permission from ref 461. Copyright 2016 Elsevier B.V.

No-wash electrochemical biosensors can effectively overcome the difficulties of heterogeneous electrochemical biosensors.[434] Up to now, various promising no-wash electrochemical strategies, including voltammetric techniques and impedimetry, have been constructed for cancer biomarker detection, and the detection performances have been largely enhanced by nanomaterials as electrode modified materials, label or signal carriers, tracers, separators and collectors, mediators, and catalysts owing to their large surface area, excellent conductivity, and catalytic activity.[435,436]

VOLTAMMETRIC NO-WASH BIOSENSORS

Cyclic Voltammetry (CV). CV is performed by varying the applied potential at a working electrode in both forward and reverse directions while monitoring the current. Through coupling with CV, Miao and co-workers[437] developed an electrochemical sensor for miRNA-21 detection in solution based on a target-mediated sandwich hybridization reaction. The target miRNA-21 first hybridized with the capture probe preconjugated onto the AuNP-modified electrode surface. After the addition of the G-rich region of detection probe-modified AuNPs, a sandwiched DNA complex was formed, and the G-rich region on the AuNP surface was transformed into a G-quadruplex structure, which can specifically interact with iridium(III) complex, thereby triggering an electrochemical signal change with methylene blue as an electron mediator. Lu *et al.*[438] subsequently reported an improved electrochemical method to sensitively analyze miRNA-182 through target-induced opening of hairpin-structured DNA probes coupled with CV, where AuNP-coated magnetic microbeads and AuNPs were used as carriers of hairpin-structured DNA probes and ferrocene was used to amplify voltammetric signal, respectively. More recently, an exciting design was published by Feng and colleagues,[439] who integrated the ECL and CV to build a multiplexed ratiometric biosensor for simultaneous detection of miRNA-21 and miRNA-141 in solution, in which the ferrocene-labeled hairpin DNA was used as CV signal tags. To quench the ECL signal tags, poly-L-lysine (PLL) was added as a bridging agent to conjugate $Ru(bpy)_3^{2+}$-doped silica and AuNPs. With target miRNA, the hybridization with the corresponding hairpin DNA would result in displacement of ferrocene from the ECL signal tags (Figure 15A). As such, the target-induced conformational changes could induce the recovery of the ECL signal and decrease the CV current of ferrocene to achieve dual-signal ratiometric detection. For protein biomarker detection, many attempts have been devoted to developing no-wash electrochemical immunosensors combined with CV.[440,441] For example, Lin *et al.*[442] displayed an electrochemical immunosensor for no-wash detection of epidermal growth factor (EGF) based on CV (Figure 15B). The authors found that the usage of oxidized glutathione to replace the BSA molecule as a locking reagent can effectively improve the detection sensitivity, owing to the elimination of steric hindrance between EGF and antibody-modified AgNPs.

Differential Pulse Voltammetry (DPV). DPV is conducted by scanning the potential with a series of pulses of fixed small amplitude (10−100 mV) and superimposing on a slowly changing base potential, where the currents of two points are measured for each pulse and the two points are recorded before the pulse application and at the end of the pulse, respectively. DPV-based electrochemical sensors have received increasing interest in detecting different analytes, such as small molecules and tumor biomarkers. As early as 2004, Kim *et al.*[443] reported on a wash-free electrochemical sensor for DNA detection at a AuNP-modified electrode through the usage of competitive hybridization reaction in combination with DPV. The target DNA can displace capture DNA and then hybridize with signaling DNA to form dsDNA, resulting in the displacement of signaling DNA from the sensor surface that can cause the electrochemical signal change. Thereafter, a no-wash DPV-based electrochemical sensing platform for the detection of DNA methylation was established by target-mediated accumulation of methylene blue in the DNA hybrid as an electrochemical signal label. The DNA methylation level was monitored *via* recording the voltammetric signal change of methylene blue at a AuNP-modified electrode, and simultaneously this sensor also could be used for the analysis of methyltransferase activity.[444] To improve the sensitivity of no-wash electrochemical biosensing methods, some effective signal amplification strategies, such as enzyme-based signal amplifica-

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tion (*e.g.*, exonuclease I or III,[434,445] nicking endonuclease,[446] DSN,[447] DNA polymerase,[448] and enzyme-free signal amplification (HCR)[449]), were introduced, and these amplified no-wash DPV-based electrochemical biosensors were successfully used for ultrasensitive detection of cancer-related DNA, miRNA, and protein. For example, an affinity-based electrochemical sensor created by GO-modified glassy carbon electrode was developed for no-wash detection of CEA by Ge *et al.*,[450] who used the aptamer as a recognition element and methylene blue as an electrochemical probe for DPV coupled with T7 exonuclease-assisted target recycling amplification (Figure 16A). Combined with conventional immunoassay, several DPV-based no-wash electrochemical immunosensors were designed for target protein. Zhao *et al.*[451] first developed a DPV-based electrochemical immunosensor for CEA through the use of nanocomposites composed of reduced GO, AuNPs, and poly(indole-6-carboxylic acid) (PICA), where reduced GO was used to enhance the conductivity of PICA and provide larger surface area for more AuNP immobilization. Ren *et al.*[452] applied a controlled release system to build a sensitive DPV-based no-wash electrochemical immunosensor for PSA by using target-induced proximity hybridization to trigger the release of the electroactive methylene blue from DNA-gated mesoporous SiO_2 (Figure 16B). Later, a similar electrochemical immunosensor was reported for the determination of PSA with acid cleavable linkage instead of proximity hybridization for controlling the release of the electron mediator thionine from AuNP-caged mesoporous SiO_2.[453] Recently, a triple signal amplification strategy composed of polymer nanospheres, Pt NPs, and DNAzyme was introduced into the electrochemical immunosensor for sensitive analysis of AFP in solution *via* measuring the DPV response change.[454] To better determine the protein marker, a DPV-based ratiometric electrochemical immunosensor was presented for CEA, where the ratio was conducted on the basis of the alterations of the electrochemical indicator signal of $K_3[Fe(CN)_6]$ and the constant internal reference signal generated between polythionine−Au and AuNP-modified electrode surface.[455] Besides nucleic acid and protein detection, the DPV-based no-wash electrochemical biosensors played a key role in monitoring various tumor-related enzyme activities. Shin and colleagues[456] first reported a simple DPV-based no-wash electrochemical biosensor for protein kinase activity, where the peptide substrate was phosphorylated to bind with the phosphate-specific receptor coated on a AuNP-modified electrode surface, thus yielding a redox current change. Based on Dpn I digestion-triggered complexation between polyadenine DNA and AuNPs, Liu *et al.*[457] explored an electrochemical strategy for monitoring DNA methyltransferase. In this case, the methylene blue-labeled DNA hairpin probe was methylated and then designed by restriction endonuclease to release a methylene blue−polyadenine DNA complex that could bind to the surface of AuNP-modified glassy carbon electrode through the interaction polyadenine and AuNPs, thus favoring an obvious electrochemical signal. To obtain a higher sensitivity for enzyme monitoring, exonuclease III or T7-assisted signal amplification was introduced into a no-wash electrochemical biosensing strategy for sensitively detecting DNA methyltransferase[458,459] and telomerase activity.[460] Furthermore, using AuNPs as methylene blue carrier, Miao and co-workers[461] designed an ultrasensitive DPV-based electrochemical biosensor for protein tyrosine kinase-7 assay in solution, in which the tyrosine kinase-7 could induce a structural transformation of sgc8 aptamer to trigger the decrease of the electroactive methylene blue molecules, thereby resulting in a reduced electrochemical signal at a AuNP-modified glass carbon electrode (Figure 16C).

Square Wave Voltammetry (SWV). In SWV, the excitation signal is made of two parts of a symmetrical square-wave pulse of amplitude and a staircase waveform of step height. The net current is centered on the redox potential and is determined by measuring the difference between the forward and reverse currents. Due to the excellent sensitivity, speed, and distinguished capability related to nonfaradic current, SWV has been used to fabricate no-wash electrochemical sensors to detect various target analytes, including small molecules, DNA, and protein. Idili *et al.*[462] demonstrated a SWV-based no-wash electrochemical detection system for target DNA based on the target-binding controlled folding-upon-binding to trigger the change of electrochemical signaling at a AuNP-modified electrode surface. Moreover, SWV-based electrochemical biosensors were also used for no-wash detection of protein biomarkers. For instance, using target-induced aptamer structure switching, two groups of Cao *et al.*[463] and Su *et al.*[464] reported a no-wash electrochemical biosensing method to sensitively test for osteopontin and thrombin by using AuNPs and AuNP-decorated MoS_2 nanosheets as electrode modified materials, respectively. Combined with conventional immunoassay, a SWV-based no-wash electrochemical immunosensor can serve as an important analytical tool for early detection of protein biomarkers *in vitro*. For example, a no-wash electrochemical immunosensor platform was proposed to measure various tumor-related protein biomarkers by direct electrochemical readout of SWV signal at a AuNP-modified electrode surface.[465,466] Later, two controlled release-based SWV electrochemical immunosensors were demonstrated for no-wash detection of squamous cell carcinoma antigen by using specific immunoreaction and pH stimuli to trigger the release of the electroactive toluidine blue and methylene blue from aminated polystyrene microsphere-caged magnetic mesoporous Fe_3O_4 and AuNP-caged mesoporous SiO_2, respectively.[467,468] Furthermore, an interesting electrochemical immunosensor for no-wash detection of CEA was described by Su *et al.*[469] In this case, the target-triggered SWV electrochemical signal change was achieved through controlling the shape of AuNPs decorated thionine−MoS_2 nanosheet nanocomposites, with thionine as the reducing agent for gold growth.

Other Voltammetric No-Wash Biosensors. Besides the above three voltammetric no-wash electrochemical biosensors, the other two voltammetric technologies, including chronocoulometry and alternating current voltammetry (ACV), were also introduced for constructing no-wash detection systems. For instance, Roberts and co-workers[470] used a chronocoulometry electrochemical sensor for PSA detection based on target-induced digestion of a peptide sequence and transduced the cleavage chemistry into an electrical signal collected at gold nanowire electrodes. Hsieh *et al.*[471] demonstrated an ACV-based polarity-switching electrochemical sensor through a AuNP-modified electrode for specific detection of single-nucleotide mismatches, in which the hybridization of perfectly matched single-nucleotide would result in a decreased output signal, whereas an increased signal from the hybridization of a single-nucleotide mismatched target, accordingly, facilitated the quantitative determination of the target. With the combination of proximity assay and immunoreaction, an ACV-based no-wash ratiometric electrochemical biosensing platform was

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explored for PSA detection.[472] This ratiometric design was implemented on the basis of target-induced formation of sandwich immunocompounds among two antibody-labeled DNA probes and the target protein. The hybridization of the two DNA molecules led to the formation of a three-armed DNA structure on the sensing interface. Along with the DNA assembly, the ferrocene was displaced from the AuNP-modified electrode surface, while the methylene blue approached the AuNP-modified electrode, giving rise to a reduced ferrocene signal and an increased MB signal for ratiometric readout.

IMPEDIMETRIC NO-WASH BIOSENSORS

Electrochemical impedance spectroscopy (EIS) depends on the measurement of the resistive and capacitive properties of materials from the perturbation of a small amplitude sinusoidal AC excitation signal. Similarly, an impedance spectrum is obtained by altering the frequency over a wide range. Combined with the EIS assay, several no-wash electrochemical biosensors have been designed to detect protein biomarkers. Based on the specific recognition between target and aptamer, Wang's group[473] and Ma's group[474] presented an EIS-based no-wash electrochemical aptasensor for quantification of CEA and thrombin, respectively. The former relied on the binding ability of GO toward ssDNA and dsDNA and on the electrochemical impedance transducer at a GO-modified glassy carbon electrode from the amplification system of an aptamer-switched bidirectional DNA polymerization reaction. The latter sensor operated upon the formation of a sandwich complex among a sulfhydryl-conjugated capture probe, a biotin-labeled reporter probe, and target thrombin coupled with a dual signal amplifier generated from the nanocomposites of AuNP-decorated GO and CoPd binary nanoparticles, where the AuNP-decorated GO exhibits excellent electron-transfer capacity and large specific surface area and CoPd shows fine catalytic activity toward H_2O_2. Moreover, an ESI-based no-wash electrochemical immunosensor from Kavosi *et al.*[475] was demonstrated for the testing of AFP. Along with the occurrence of the immunoreaction between AFP and corresponding antibody, an increased ESI response was recorded because of the hindered electron-transfer reaction on the AuNP-modified electrode surface from the binding of target AFP. To enhance the ESI signal, the author also used AuNP/polyamidoamine dendrimer nanocompounds to increase the electrode surface area and conductivity for a highly efficient Au electrode sensing surface.

OUTLOOK OF ELECTROCHEMICAL NO-WASH BIOSENSORS

Over the past few decades, the great potential of electrochemical no-wash biosensors in bioanalytical applications has come to fruition and has produced numerous advances because of their high sensitivity, low cost, and easy operation. However, there are still some challenges that need to be addressed before the applications of electrochemical methods for clinical detection of cancer biomarkers are realized. The current electrochemical assays require the electrode modification, which makes the operation complex and difficult to generate reproducible results. In addition, the excellent specificity to accurately differentiate target analytes in complicated clinical samples is another challenge to ensure the reliability of detection. Besides, the nonspecific adsorption of nontarget analytes in complex matrices on transducing sensor surfaces can reduce the signal-to-noise ratio and lead to a false detection signal. Moreover, although nanomaterials have been widely applied to improve the analytical performance of electrochemical biosensors, mass-scale controllable synthesis of nanomaterials and the nonspecific adsorption of background impurities on the nanomaterial surface should also be a matter of concern. Finally, although some multiplexed electrochemical sensors have been reported, there is still plenty of room for improvement. Therefore, further investigation may focus on designing electrochemical biosensors without any electrode modification, optimizing the synthesis of nanomaterials, mitigating nonspecific adsorption, improving the multiplexing detection potential through exploring signal recognition elements with multiple recognition sites or with different distinguishable electroactive indicators, as well as designing portable miniaturized electrochemical devices.

LIGHT-SCATTERING-BASED NO-WASH BIOSENSORS

It is well-known that incident light with a greater wavelength than a nanostructure's size can cause the coherent collective oscillation of electrons at the surface of metallic nanostructures, which can produce strong electromagnetic scattering. Generally, light scattering can be classified into elastic and inelastic light scattering. During the elastic scattering process, energy transfer will not occur, such as Rayleigh scattering and Mie scattering. On the contrary, in an inelastic scattering process, energy transfer is required, as in the case of Raman scattering. The scattered light intensity can be measured by using the corresponding optical techniques, such as Raman spectroscopy and Rayleigh scattering spectroscopy, *etc.* It has been reported that AuNPs show extremely strong light-scattering ability at the plasmon-resonance wavelength, which is several orders of magnitude higher than that of nonmetallic materials of the same size and over a million times greater than fluorescent molecules. This ultrastrong light-scattering property makes AuNPs one of the most widely used optical labels in light-scattering-based sensing technologies. To date, a majority of biosensors have been developed for chemical and biological analysis using the phenomenon of elastic light scattering of AuNPs, such as resonance light-scattering correlation spectroscopy (RLSCS)-based sensors and dynamic light scattering (DLS)-based assays. Moreover, for inelastic scattering, Raman scattering-based biosensing methods have also been extensively proposed for target analyte detection by directly measuring the scattered light from specific nanoassemblies or the enhanced scattered light from the Raman active dye molecules located on the surface of nanomaterials, as for metal nanoparticles. In this section, we will focus on the latest developments in light-scattering-based sensing platforms for the applications of *in vitro* detection of cancer biomarkers with special emphasis on wash-free biosensors.

RLSCS-BASED NO-WASH BIOSENSORS

RLSCS, as a single-molecule detection method, is based on the determination of the resonance light-scattering fluctuations from the Brownian motion of single NPs in a small volume (<1 fL). The scattering light fluctuations can be recorded using a sensitive single-photon counting device. Due to their strong resonance light scattering, metallic NPs can act as sensitive optical labeling probes for the development of RLSCS-based sensors. As early as 2007, the RLSCS-based no-wash biosensors using AuNPs as labels have been used to detect DNA hybridization by measuring the change in translational

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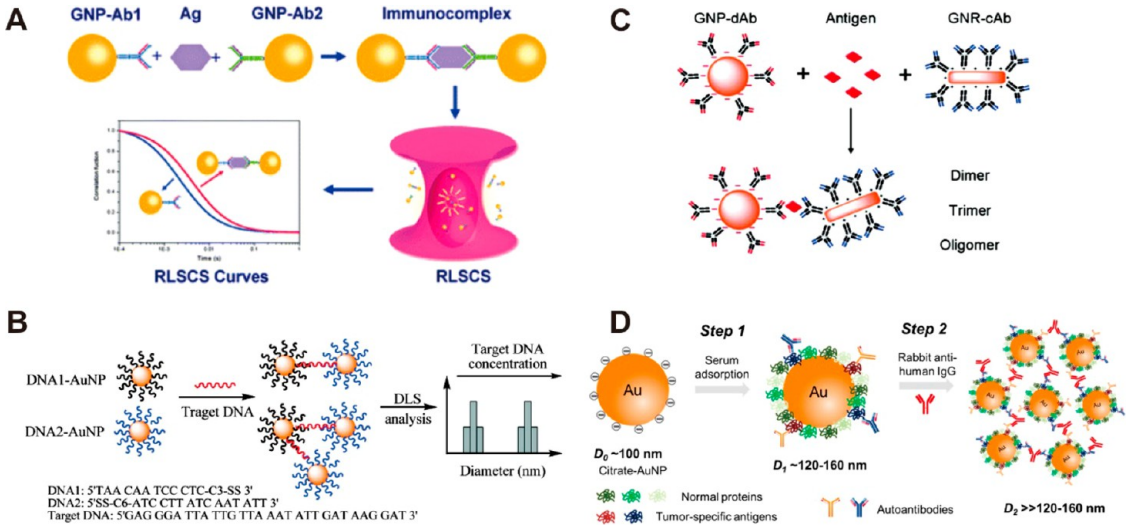

Figure 17. (A) RLSCS-based no-wash immunosensor for AFP. Reprinted with permission from ref 477. Copyright 2011 The Royal Society of Chemistry. (B) AuNP-based no-wash DLS detection for DNA. Reprinted with permission from ref 481. Copyright 2008 American Chemical Society. (C) DLS-based no-wash immunosensor for PSA. Reprinted with permission from ref 482. Copyright 2008 American Chemical Society. (D) Two-step no-wash DLS immunosensor for PSA. Reprinted with permission from ref 486. Copyright 2015 American Chemical Society.

time.[476] After that, several RLSCS-based sandwich immunosensors were fabricated for no-wash detection of AFP (Figure 17A) and PSA using AuNPs and AgNPs as probes, respectively.[477,478] In addition, using anisotropic AuNRs as labeling probes, Zhang *et al.*[479] presented a RLSCS-based no-wash sandwich immunosensor for AFP by characterizing the changes of translational diffusion behaviors of single AuNRs and immunocomplexes (AuNR-AFP-AuNR dimer). Recently, Hu *et al.*[480] proposed a no-wash RLSCS-based biosensor to monitor the activity of caspase 3. In this design, the AuNP dimer was preprepared through utilization of the biotin−streptavidin interaction between streptavidin-conjugated AuNPs and biotinylated peptide-labeled AuNPs. The presence of target caspase 3 could digest the peptide bridge, resulting in the separation of AuNP dimers, further causing RLSCS signal change.

NANOPARTICLE SIZE CHANGE-BASED NO-WASH BIOSENSORS

DLS, based on the phenomenon of light scattering, has become a powerful analytical tool for characterizing the particle size of materials from 0.5 nm to 6 mm in solution. Due to their higher light-scattering abilities over most biological samples, metal NPs, such as AuNPs or AgNPs, have been routinely used as signal amplification probes in DLS-based biosensing platforms. Compared with other analytical technologies, DLS-based biosensors provide many advantages, including high sensitivity and wash-free detection in solution. These features make DLS-based sensors one of the most competitive analysis platforms in no-wash detection. Recently, various DLS-biosensors have been investigated based on analyte binding-induced AuNP aggregation or individual GNP size change upon analyte binding to sensitively measure cancer-related biomarkers such as DNAs and proteins, with excellent sensitivity.

A significant contribution in this field was from Dai and coworkers,[481] who first reported a highly sensitive and no-wash DLS-based method for target DNA detection in solution (Figure 17B). In this sensor, two DNA-functionalized AuNP probes were prepared and hybridized with complementary target DNA in solution, which could cause the formation of dimers, trimers, and larger aggregates of AuNPs. The produced AuNP aggregates would result in an increase in AuNP size, which can be easily detected by DLS analysis. Using a similar strategy, other researchers also developed AuNP-based DLS biosensors for target DNA analysis based on target-induced increase or decrease in AuNP size.[28] Analogous to DNA hybridization-induced AuNP aggregation, the specific immunorecognition of antigen and antibody was employed to adjust the nanoparticle aggregation. For instance, Liu *et al.* reported a wash-free DLS-based immunosensor for PSA achieved by the formation of an immunocomplex among antibody-labeled AuNPs, antibody-labeled AuNRs, and target PSA to induce the generation of dimers, oligomers, or aggregates between AuNPs and AuNRs, which can lead to an increase in AuNP size (Figure 17C).[482] Subsequently, other tumor-related proteins, such as CA125, CEA, CA19-9, prostatic acid phosphatase (PAP), and AFP, were determined by using a no-wash DLS-based immunosensor.[483−485] The above-mentioned DLS-based immunosensors are based on a one-step "mix-and-measure" model. Recently, Zheng *et al.*[486] reported on a two-step AuNP-enabled no-wash-based immunosensor for PSA. In this design, the tumor-specific antigen and normal proteins first compete to adsorb onto the citrate-modified AuNPs to form a protein corona, and then the adsorbed tumor-specific antigens further adsorb tumor-specific autoantibodies (human IgG) to the AuNP surface, which can cause the first increase in AuNP size. Then, with the addition of antihuman IgG, a large AuNP cluster is formed based on the binding between antihuman IgG and IgG adsorbed on AuNP suface, which can give rise to a second increase in AuNP size (Figure 17D). By recording the two AuNP sizes in the first and second step, the ratio of two-step AuNP sizes is used to quantify target analytes.

RAMAN SCATTERING-BASED NO-WASH BIOSENSORS

Raman scattering is an inelastic scattering of photons from a quantized molecular vibrational signature. Raman scattering

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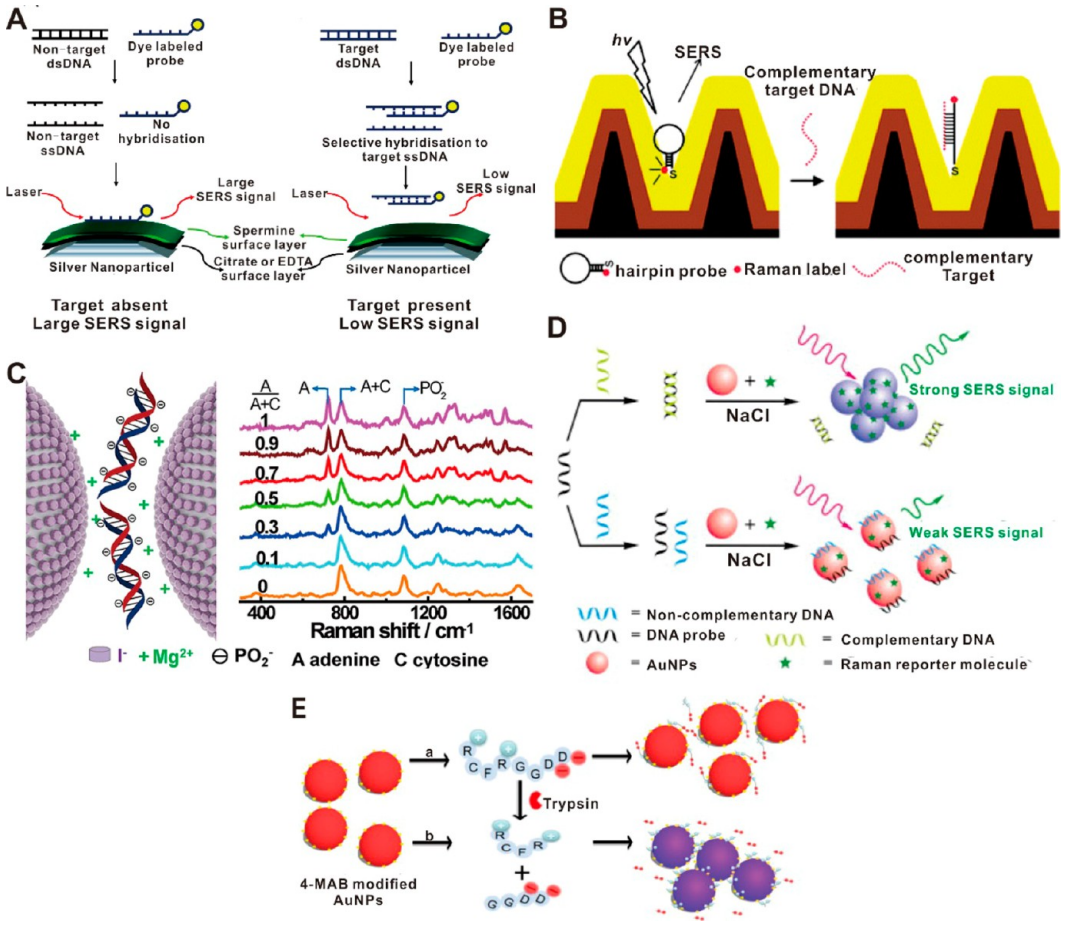

Figure 18. (A) SERS detection assay based on the affinity differences of dsDNA and ssDNA toward AgNP surface. Reprinted with permission from ref 492. Copyright 2009 American Chemical Society. (B) MB-based DNA detection on a triangular-shaped nanowire substrate. Reprinted with permission from ref 496. Copyright 2013 the Owner Societies. (C) SERS detection of DNA with iodide-modified AgNPs. Reprinted with permission from ref 506. Copyright 2015 American Chemical Society. (D) No-wash SERS-based biosensing platform for DNA detection of salt-induced aggregation of unmodified AuNPs. Reprinted with permission from ref 508. Copyright 2012 Elsevier B.V. (E) SERS-based enzyme assay for trypsin with peptide-mediated aggregation. Reprinted with permission from ref 514. Copyright 2014 Elsevier B.V.

intensity is sensitive to vibrational modes and provides feasibility for fingerprint sensing of target molecules. However, conventional Raman scattering is very weak, and only high concentrations of Raman dye molecules can be detected, seriously restricting the application of this technique. In the early 1970s, an exciting phenomenon of surface-enhanced Raman scattering (SERS) was first reported by researchers. Since then, Raman scattering-based sensing technologies have been the focus of intensive development. Previous studies have shown that Raman scattering intensity can be enhanced dramatically by metal nanomaterials based on electromagnetic field enhancement from LSPR and chemical enhancement through charge transfer, and the enhancement extent reaches up to 10^{14} in comparison with normal Raman scattering.[487] As such, SERS provides detection sensitivity down to a single-molecule. Moreover, SERS also exhibits several other advantages, including simplicity, rapidity, good selectivity, excellent reproducibility, *in situ* noninvasive detection, rich spectral fingerprint signatures, as well as multiplexing analysis ability.[488,489] Additionally, SERS spectroscopy can be conducted in solution, offering the possibility of no-wash detection to allow for the identification of components in a sample without the need for additional separation. Thus, SERS has increased in popularity as an important analytical tool in biosensor platforms. So far, various types of SERS-based biosensors have been demonstrated for sensitive chemical and biological analysis.[490,491] Among these reported SERS-based methods, most design strategies exploit the adsorption of target molecules or Raman active probes onto the roughened metal surface or film to generate an enhanced Raman scattering signal. Although these SERS-based analysis technologies involve nanotextured surfaces to display superb sensitivity for target analytes, they are performed in a heterogeneous format, requiring multiple washing steps to remove excessive Raman labels or labeled biomolecules. Thus, to avoid repeated washing and simplify the assay procedure, the development of SERS-based no-wash biosensors has become an important focus for the field. Presently, SERS-based biosensing strategies have been demonstrated as a useful tool for biomedical detection and trace analysis. These typical SERS-based designs were achieved by controlling SERS enhancement on the basis of target-triggered adsorption/release of Raman labels, target-induced aggregation/anti-aggregation of metallic NPs, as well as target-mediated self-assembly of metallic NPs. In this section, we will review the latest advances of applications of SERS-based no-wash biosensors in early diagnosis of cancer *in vitro*.

Target-Triggered Adsorption/Release of Raman Labels. Metallic NPs, such as AuNPs and AgNPs, are the most

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widely used SERS substrates because of their easy synthesis and strong enhancement capacity for Raman scattering. As mentioned above, ssDNA shows a higher affinity for the surface of metallic NPs than dsDNA. Thus, the first strategy for SERS-based no-wash biosensors for target DNA relies on the adsorption difference of ssDNA and dsDNA to metallic NPs (Figure 18A). Through using a SERS probe modified with complementary ssDNA to adsorb onto the metallic NP surface, an increased SERS signal is obtained in the absence of target. After the formation of dsDNA with the hybridization of target DNA and complementary ssDNA, the SERS probe modified with complementary ssDNA becomes separated from the metallic NP surface due to the detachment of dsDNA, thereby resulting in a deceased SERS signal. Using this sensing method, several SERS-based no-wash biosensors have been developed for DNA analysis.[492−494] When different SERS probes are employed to label different oligonucleotides, this sensing strategy provided a potential for multiplexed no-wash detection.[495] Another strategy for the development of SERS-based DNA sensors depends on the control of the closed or opened state of SERS probe-tagged DNA hairpin probe (Figure 18B). In the absence of target DNA, the closed stem loop configuration of hairpin probe maintains the SERS probe in proximity to metallic NPs, inducing a strong Raman signal. With the hybridization of a complementary target DNA and the closed hairpin probe, the stem loop configuration is opened, causing the separation of the SERS probe from the metallic NP, thus leading to a reduced SERS signal. Based on this strategy, Wang *et al.*[496] reported on a SERS-based biosensor for no-wash detection of the *Ki-67* gene as a critical breast cancer biomarker. Additionally, other researchers have also developed several similar SERS-based biosensor approaches for the assay of disease-related DNA biomarkers.[497,498] To achieve better diagnosis of cancer, simultaneous detection of multiplex DNA biomarkers has also been performed through use of SERS-based biosensors with different SERS probe-tagged DNA hairpin configurations. For example, Wang and co-workers[499] first demonstrated a multiplexing SERS-based biosensing technology to simultaneously measure two breast cancer genes, *erbB-2* and *ki-67*. Recently, this group also presented a multiplexed SERS-based sensing technology for multiplex detection of miRNA in solution.[500] This sensor was based on an inverse strategy, where the presence of target miRNA would cause the SERS probe to approach the surface of silver-coated gold nanostars to generate an enhanced SERS signal owing to the formation of a DNA hairpin structure. Besides nucleic acid detection, the SERS-based sensing platform is also considered a powerful analytical technique for protein and enzyme assays. Cho *et al.* described a SERS-based sensor for thrombin by applying target-triggered displacement of SERS probe-labeled aptamer from the AuNP surface, which results in a decrease in SERS signals.[501] In addition, Moore and colleagues[502] designed a SERS-based no-wash sensing strategy to monitor cancer-related enzyme activities. This sensor was designed by fabricating masked enzyme substrates without any SERS signal. Enzyme-induced substrate cleavage to release the SERS probe to the AgNP surface caused an intense SERS signal that was proportional to enzymatic activity. Finally, the proposed sensor was successfully applied to determine the activities of 14 enzymes including lipases, esterases, and proteases and exhibited potential for multiplexing analysis of enzyme activities within a single cell.

Target-Induced Aggregation/Anti-Aggregation of Metallic Nanoparticles. Although the enhanced SERS signal based on a single metallic nanoparticle surface has been successfully employed for cancer biomarker detection, the enhancement ability of single metallic nanoparticles for Raman scattering is limited since the SERS signal of individual nanoparticles is rather weak. To obtain a higher SERS signal, an improved method was recently proposed by using the aggregation of AuNPs or AgNPs. This is due to plasmon coupling between nanoaprticles in close proximity, which can confer enormous electromagnetic field enhancements at junction sites or SERS "hot spots". It has been reported that Raman scattering can be significantly enhanced up to $10^{14}-10^{15}$ with the aggregation of nanoparticles. As such, controlling the aggregation of AuNPs or AgNPs is of considerable interest to the development of SERS assays for ultrasensitive biomarker detection. Several strategies have been introduced to achieve the aggregations of nanoparticles, including chemically driven aggregation with/without an aggregating agent (such as positively charged materials or salt) and peptide-induced aggregation.

It is known that DNA with a highly negatively charged phosphate backbone is very hard to directly bind to the surface of metallic NPs due to the repulsion with the negatively charged surface. To overcome this problem, positively charged spermine has been proposed to neutralize the phosphate backbone of DNA and aggregate the nanoparticles. Graham *et al.* first demonstrated the proof of concept in 2000, where a no-wash SERS-based sensor was acquired for labeled DNA analysis on the basis of spermine-induced AgNP aggregation to enhance Raman scattering.[503] After that, many similar SERS-based biosensors were constructed for other labeled DNA assays.[504] Nevertheless, the synthesis of labeled DNA strands is complicated and costly. Therefore, it is very attractive to perform direct SERS analysis of unmodified DNA. To realize this goal, Guerrini *et al.*[505] recently designed an enhanced SERS-based sensing platform for direct measurement of DNA by employing spermine-modified AgNPs as SERS probes, where spermine can act as a AgNP stabilizer and aggregation agent. Based on the similar concept, Xu *et al.*[506] also displayed an improved SERS-based biosensing method for direct analysis of unlabeled DNA by applying magnesium sulfate to replace spermine as an aggregation agent to trigger the aggregation of iodide-modified AgNPs (Figure 18C). Additionally, this team also used the same iodide-modified AgNPs to enhance SERS-based biosensors for the label-free measurement of five target proteins.[507] Salt-induced aggregation to enhance Raman scattering is another strategy for building sensitive SERS-based no-wash biosensors. As mentioned above, ssDNA can better stabilize AuNPs compared with dsDNA. By taking advantage of this observation, Yi *et al.*[508] proposed a no-wash SERS-based biosensing platform for target DNA assay in solution by employing a salt-induced aggregation of unmodified AuNPs to amplify the SERS signal (Figure 18D). Later, the method was extended to detect other analytes, such as proteins[509] and small molecules,[510] by using the ssDNA to selectively bind the analytes. For instance, Wen and colleagues[509] designed a SERS-based no-wash sensor to trace HCG *via* employing salt-induced aggregated AgNPs as a catalyst and Raman scattering enhancer, whereas, with an aggregating agent, the Raman spectra are often inconsonant and the intensity ratios between the SERS bands fluctuate, leading to poor reproducibility, further restricting the wide-

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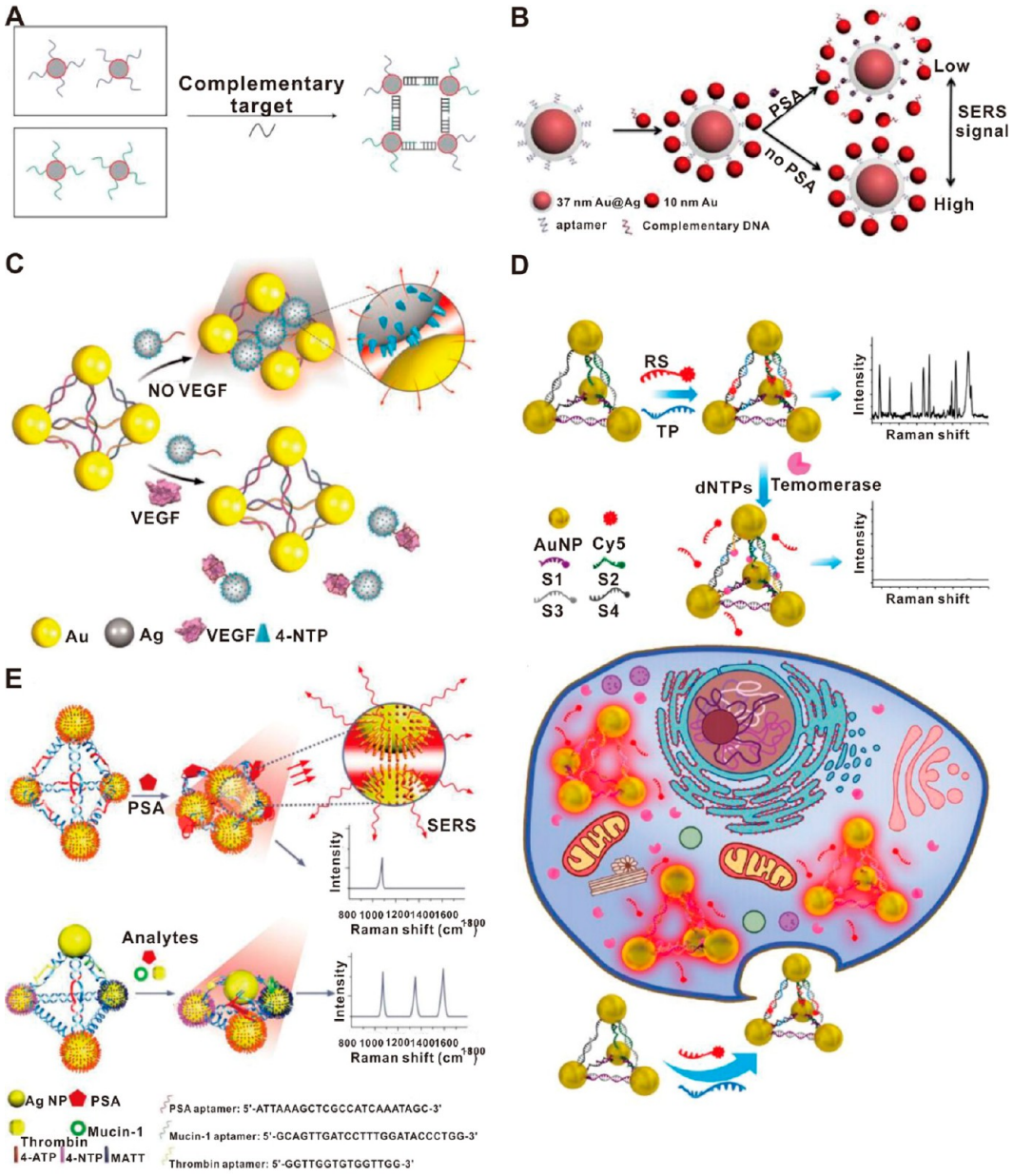

Figure 19. (A) Sandwich hybridization-based SERS using Raman dye-functionalized DNA−AgNP conjugates. Reprinted with permission from ref 517. Copyright 2008 Macmillan Publishers Limited. (B) SERS method for PSA detection based on the aptamer directed core−satellite assemblies. Reprinted with permission from ref 523. Copyright 2014 The Royal Society of Chemistry. (C) SERS detection of VEGF based on self-assembled Ag ornamented−Au pyramid superstructure. Reprinted with permission from ref 526. Copyright 2015 Elsevier B.V. (D) Pyramid-based intracellular SERS analysis of telomerase in cell extracts and in live cells. Reprinted with permission from ref 527. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA. (E) DNA frame-mediated Ag pyramid self-assembly based SERS analysis for multiplexed protein biomarkers. Reprinted with permission from ref 528. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA.

spread application of these strategies. To address this issue, Panikkanvalappil *et al.*[511] explored another SERS-based sensing method for cancer cell DNA based on the usage of AgNPs at high concentrations to produce aggregated AgNPs without any aggregating agents to generate highly reproducible SERS spectra. Ascribing to the change in the chemical environment generated from nucleobase lesions in cancer cell DNA and the subsequent variation in nearby electronic cloud during the dehydration-driven conformational changes, apart from chemically driven aggregation, peptide-induced aggregation has been introduced for enhancing SERS signal in enzymatic activity monitoring. Peptides with many arginine residues can induce metallic NP aggregation and promote SERS of Raman reporters because of a high isoelectric point, while with the hydrolysis of these peptides into fragments by target enzymes conversely decreases the SERS signal owing to the weak interaction of fragments with metallic NP surfaces (Figure 18E). Through this strategy, several facile SERS biosensors for trypsin and thrombin were constructed.[512−514] Moreover, hairpin DNA structure can stabilize the metallic NP and prevent aggregation, while the degradation of hairpin DNA substrate probes using target enzyme leads to deprotection of the metallic NP and subsequent aggregation to generate strong plasmonic coupling, which in turn amplifies SERS signal. Using this approach, Wang *et al.*[515] developed a sensitive analysis involving DNA demethylation with demethylase. Very recently, another

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aggregation approach was reported by Fazio *et al.*,[516] who applied radiation pressure to locally push AuNRs and induce their aggregation for improving SERS detection signal in biomolecular detection. This sensor exhibited a high sensitivity in detecting phenylalanine, bovine serum albumin, and lysozyme and had potential for the detection of other protein biomarkers.

Target-Mediated Self-Assembly of Metallic NPs. Although nanoparticle aggregation can largely improve the SERS detection signal related to individual NPs, uncontrollable aggregation results in signal heterogeneity with low reproducibility of the SERS assay. Moreover, the aggregation process of metallic NPs is dynamic and time dependent, which seriously affects the accuracy and reliability of aggregation-based SERS sensing strategies. Therefore, controllable assembly of metallic NPs is critical to build highly reproducible and reliable SERS-based sensors. Currently, many controllable assemblies with tunable gaps, including dimers, trimers, pyramids, chains, as well as core−satellite assemblies, have been reported for improving electromagnetic fields and consequently amplifying the SERS signals. Through design of these assemblies, a large number of highly sensitive SERS-based no-wash biosensors have been developed to determine various cancerous biomarkers, including DNA, miRNA, proteins, and enzymes.

One general approach for achieving controllable nanoassembly depends on DNA/aptamer hybridization-triggered assembly of metallic NPs. Graham *et al.*[517] first used this method to design an enhanced SERS-based nanosensor for target DNA detection, where target DNA hybridization can trigger the self-assembly of dye-coded AgNPs that produce an electromagnetic enhancement to amplify the SERS signal (Figure 19A). Subsequently, a multitude of improved SERS-based no-wash biosensors were fabricated for DNA analysis in solution by designing various assemblies, like AuNP assemblies,[518] Ag nanoprism-AuNP assemblies,[519] Au-Ag nanomushrooms,[520] and Ag@Au core−shell NP assemblies.[521] Moreover, Zhang *et al.*[522] described a multiplexed SERS-based no-wash biosensor for simultaneous detection of multiple DNAs in one sample. This multiplexing sensor was established by target-induced self-assembly of mixed DNA-functionalized AgNPs and Raman dye/DNA-conjugated AgNPs, yielding an increased SERS signal for different Raman dyes. Thus, by recording the corresponding SERS spectra from different Raman dyes, multitarget DNA detections were accomplished. Besides DNA-based nanoassemblies for target analysis, aptamer-based nanostructures exhibit extensive applications in constructing SERS-based no-wash biosensors. A general design strategy for aptamer-based SERS sensors is based on the following principle: In the absence of a target, aptamer-based nanoassemblies are formed to generate an increased SERS signal due to the hybridization of aptamer and complementary DNA, whereas in the presence of target, the specific binding of aptamer with target triggers disassembly of the nanostructure, resulting in a decrease of SERS signal (Figure 19B). Through this method, several tumor protein biomarkers, including PSA,[523] AFP,[524] and MUC1,[525] were successfully determined by target-induced disassembly of AuNP-Au@Ag core−shell NP core−satellite nanoassemblies, AgNP trimers, and AgNP-AuNR core−satellite nanostructure, respectively. Recently, through construction of pyramid superstructures to enhance the SERS signal, several ultrasensitive SERS detection methods were reported. For instance, Zhao *et al.*[526] fabricated a self-assembled AgNP ornamented−AuNPs pyramid nanosuperstructure for VEGF detection based on a specific VEGF aptamer and its partial complementary sequence, in which the aptamer-modified AgNPs could combine with the pyramid structure to produce a strong SERS signal in the absence of VEGF. On the contrary, the binding of aptamer and VEGF could trigger the release of AgNPs from the scaffold of the Au pyramid, thus resulting in a low SERS signal (Figure 19C). Subsequently, Xu and co-workers[527] presented a DNA-driven self-assembling AuNP pyramid encoding a Cy5-modified DNA sequence to detect telomerase (Figure 19D). When the target telomerase is present, the primer is extended and replaces the inner Cy5-embedded DNA sequence, causing a decrease in SERS signal and simultaneous recovery of fluorescent signal. This biosensing strategy offers an opportunity for intracellular telomerase activity monitoring. Moreover, Xu and colleagues[528] demonstrated an aptamer-driven self-assembled AgNP pyramid for simultaneous and ultrasensitive detection of multiplexed protein markers (Figure 19E). In the presence of target biomarkers, the specific biorecognition between the biomarker and corresponding aptamer alters the 3D spatial geometries of the pyramids and gives rise to a shorter gap length between adjacent AgNPs, which can significantly enhance SERS signal. This biosensor successfully measured three protein biomarkers including PSA, thrombin, and MUC1.

OUTLOOK OF LIGHT SCATTERING-BASED NO-WASH BIOSENSORS

Compared with adsorption-based biosensors, light scattering-based detection techniques exhibit a substantially higher sensitivity. In the past few decades, a plethora of highly sensitive detection strategies on the basis of light scattering of noble metal nanoparticles have been reported, and significant progress has been gained. Nevertheless, several challenges remain before their routine use in clinical diagnosis. For RLSCS- and DLS-based assays, the major challenge is the quality and stability of the AuNP, which is the most critical factor for these two assays. Additionally, the AuNP probes are prepared through weak chemical bonds or physical absorption, where the attached target recognition probes can be easily displaced from the AuNP surfaces by biothiol molecules in biological samples, further reducing the specific binding activity of AuNP probes. Besides, the strong nonspecific interactions between other biomolecules in complex sample matrices with AuNP probes may disrupt the light scattering of AuNP probes in the solution, which results in type I error or high background signal. Moreover, these methods also depend on large and costly instruments to complete data acquisition, analysis, and processing. Finally, although these two methods present high sensitivity in bioanalytical application, the multiplexing analysis capability is still to be exploited. Thus, further research should concentrate on dealing with these issues to promote the realization of commercial products for clinical diagnostics.

For SERS-based no-wash biosensors, despite some progress in the application of SERS for biomarker detection, there are still several challenges to overcome. First, preparation of highly sensitive, stable, easy-to-operate, controllable, scalable, and reproducible SERS-active nanoprobes remains a challenge, which greatly constrains SERS application in routine clinical detection. The SERS enhancement is highly dependent on active nanostructures, especially with respect to the uniformity, controllability, and reproducibility of the formed nanogaps and hot spots in the nanostructures. Second, the Raman dyes are conjugated onto the nanoparticle through physical absorption

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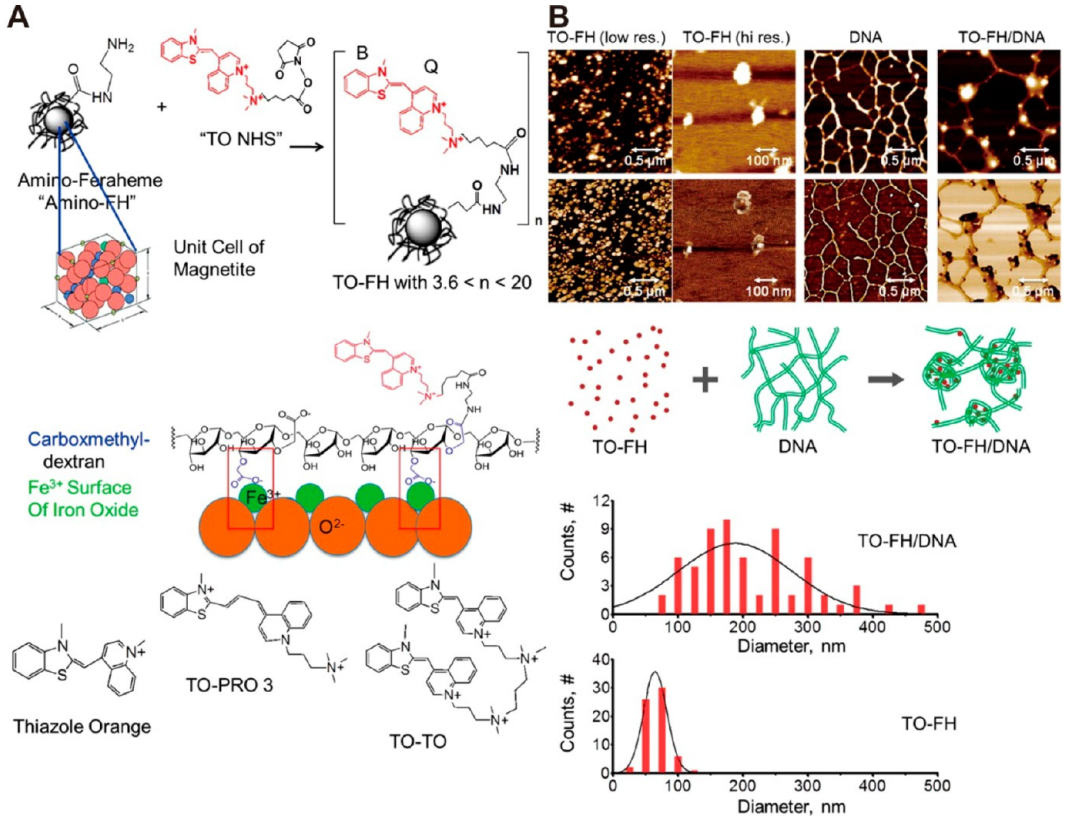

Figure 20. Schematic representation of fluorochrome-functionalized MNPs for DNA detection by microaggregate formation *in vitro*, for imaging the DNA of necrotic cells in culture and for imaging the DNA of a tumor treated with a chemotherapeutic agent. Reprinted with permission from ref 541. Copyright 2013 American Chemical Society.

or S−Au bond, which may be detached from the surface of particles in the presence of thiolated species that lead to a decreased SERS signal. Third, the SERS-active nanoprobes should be stable against aggregation in practical applications. Fourth, the currently reported SERS-based biosensors require greater quantitative resolution, particularly in complex biological samples. Fifth, the SERS-based assays with ultrahigh sensitivity still require the support of large complicated instruments and rely on SERS-mapping technologies. Although some hand-held and portable Raman spectrometers have recently been developed, the sensitivity is limited, with much room for improvement. Finally, although recent advancements have witnessed the application of SERS-based approaches for detecting multiple targets from patient samples, the overlap of Raman spectra from different dye molecules needs to be addressed to avoid the interference between dyes. Further studies should focus on fabricating of highly sensitive, controllable, and reproducible SESR-based biosensors, expanding the multiplexing capability, and designing easy-to-use and sensitive miniaturized instruments.

MAGNETIC NO-WASH BIOSENSORS

Magnetic nanoparticles (MNPs) have already been intensively applied in the field of biomedicine, including magnetic resonance imaging, drug delivery, cell separation, and hyperthermia treatment, *etc.*[529,530] Additionally, MNPs have also been involved in developing various sensors using electrochemical, optical, or piezoelectric assay models.[531] Nonetheless, in recent years, magnetic biosensor using MNPs as a signal output label has attracted widespread attention in the field of analytical chemistry. Compared with other signal reporters, the use of MNPs as biosensing labels provides several major advantages. MNPs are easy to manipulate under the applied magnetic field. Additionally, unlike electrical or optical sensing technologies, the negligible magnetic background signals of biological samples result in a high signal-to-noise ratio in magnetic sensing that can largely improve the analytical sensitivity. These prominent features make magnetic biosensors an excellent detection platform to detect nucleic acids, proteins, enzymes, and even cells.[532] Among these magnetic sensors, no-wash magnetic sensing methods have received more attention due to their outstanding advantages over heterogeneous sensors. Currently, no-wash magnetic biosensors depend on one of the two sensing mechanisms: One is based on the direct readout of magnetic signal from MNPs, and the other relies on the measurement of optical signal of MNPs under magnetic agitation.[533] For the former method, the frequently detected magnetic signals in magnetic biosensors mainly involve magnetic permeability, magnetic susceptibility, as well as magnetic relaxation.[534] However, in the no-wash detection of cancer biomarkers, magnetic biosensors mainly depend on the measurement of magnetic relaxation. For the latter method, optomagnetic (magneto-optic) detection utilizes the linked magnetic and intrinsic optical anisotropies of the MNPs or clusters of MNPs, so that the scattering and absorption of light by MNPs and clusters of MNPs change accordingly to magnetically induced orientation changes of MNPs in solution with an applied oscillating magnetic field.[535,536] These optomagnetic approaches exhibit a larger detection signal, greatly simplifying the setup and eliminating the need for polarizers. As an alternative to magnetic signal-based sensors

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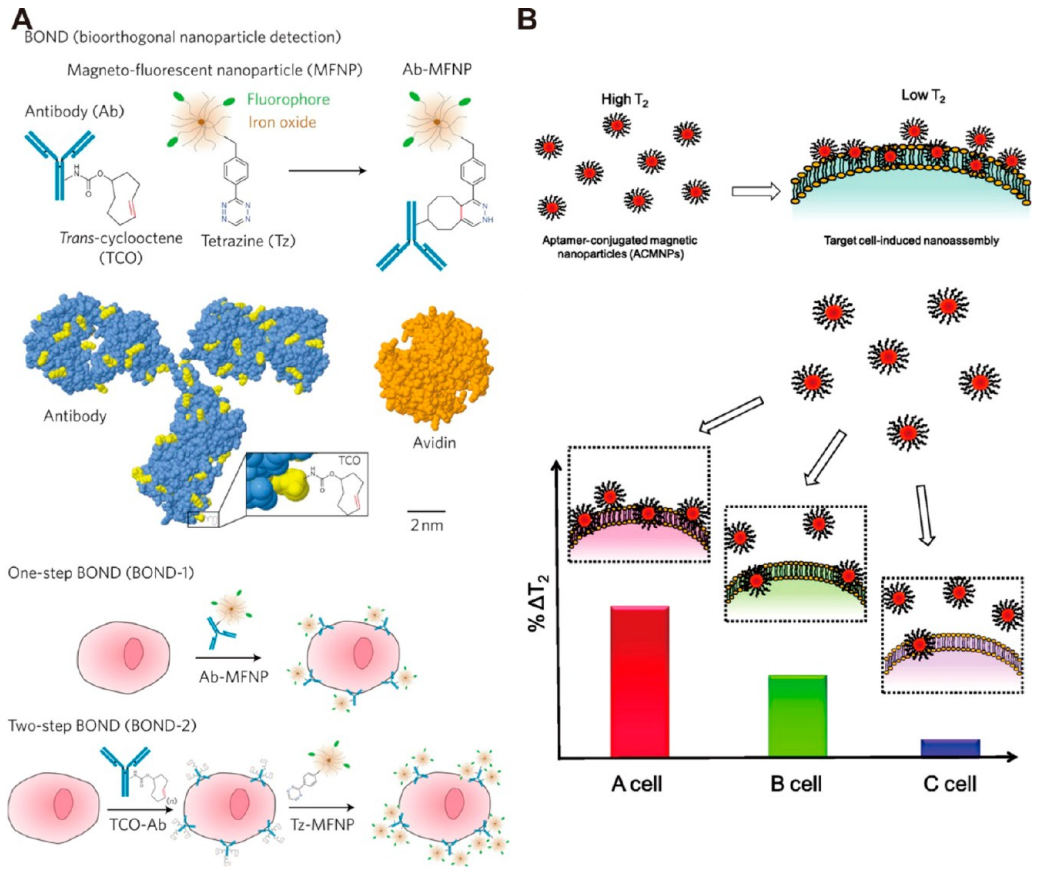

Figure 21. (A) Schematic diagram of application of BOND for one-step (direct) and two-step targeting of nanoparticles to cells. Reprinted with permission from ref 557. Copyright 2010 American Chemical Society. (B) Magnetic nanosensor for cancer cell detection and pattern recognition. Reprinted with permission from ref 558. Copyright 2012 American Chemical Society.

for target detection, optomagnetic readout strategies have also gained great interest in biosensing. Among these developed biosensors, no-wash optomagnetic assays have been proposed for *in vitro* detection of tumor-related biomarkers. This section mainly focuses on various MNP-based no-wash biosensors, which includes magnetic relaxation switching (MRSw)-based and optomagnetic biosensors.

MRSw-Based No-Wash Biosensors. MNPs can effectively increase the magnetic resonance signal of protons from surrounding water molecules. Previous studies demonstrated that the aggregation of MNPs can give rise to the coupling of magnetic spin moments and produce strong local magnetic fields. The resulting strong local magnetic field incoherence can accelerate the dephasing of surrounding water protons, arousing a corresponding decrease of transverse or spin−spin relaxation times (T_2). Therefore, the MNP aggregation or disaggregation can be detected through the change in proton relaxation times (ΔT_2), which can be detected by nuclear magnetic resonance (NMR), magnetic resonance imaging (MRI), or relaxometry. Thus, the proton relaxation times of T_2 can be applied for developing MRSw-based biosensing applications.[537] MRSw-based biosensors are a simple "mix-and-measure" analytical method, which does not require any washing step. Moreover, the assay occurs in solution and shows faster binding kinetics than a solid assay, thus necessitating only a short assay time. Up to now, various targets, including small molecules, nucleic acids, proteins, enzymes and large biological objectives (*e.g.*, bacteria, viruses and cancer cells), have been successfully detected in solution by MRSw-based no-wash biosensors.[538] According to the target biomarker size, MRSw-based no-wash biosensor can take two forms. For small target analytes, MRSw-based no-wash biosensors are based on the changes of MNP state in solution. Here, the specific binding between the target and recognized molecules can induce the aggregation/disaggregation of MNPs in solution, which can cause corresponding T_2 changes, thereby facilitating the determination of small target analytes. As early as 2001, Josephson *et al.*[539] first developed a no-wash MRSw-based sensor for oligonucleotide analysis by using the target DNA-hybridization-triggered cross-linking aggregation of MNPs. Later, an improved MRSw-based biosensor for *in vitro* DNA monitoring was reported by designing fluorochrome-functionalized MNPs to bind target DNA through fluorochrome-mediated interactions to induce the formation of micro-aggregates, which can lead to changes in T_2 (Figure 20).[540,541] Recently, Jiang's team[542] reported an exciting MRSw-based biosensing approach for miRNA assay in solution. The smart design was performed by using DSN enzyme to selectively degrade the DNA-MNP conjugates (30 nm) after the hybridization between target miRNA and DNA-MNP conjugates to release 30 nm MNPs and meanwhile recycle the intact miRNA to attend to the next cycle and further enhance T_2. For protein detection, Kim *et al.*[543] first reported a MRSw-based biosensing platform for HCG in solution through analyte-induced aggregation of MNPs with the assistance of antigen−antibody interaction. Later, several similar MRSw-based no-wash immunosensors were established for autoantibody[544] and thrombin.[545] Meanwhile, a MRSw-based multi-

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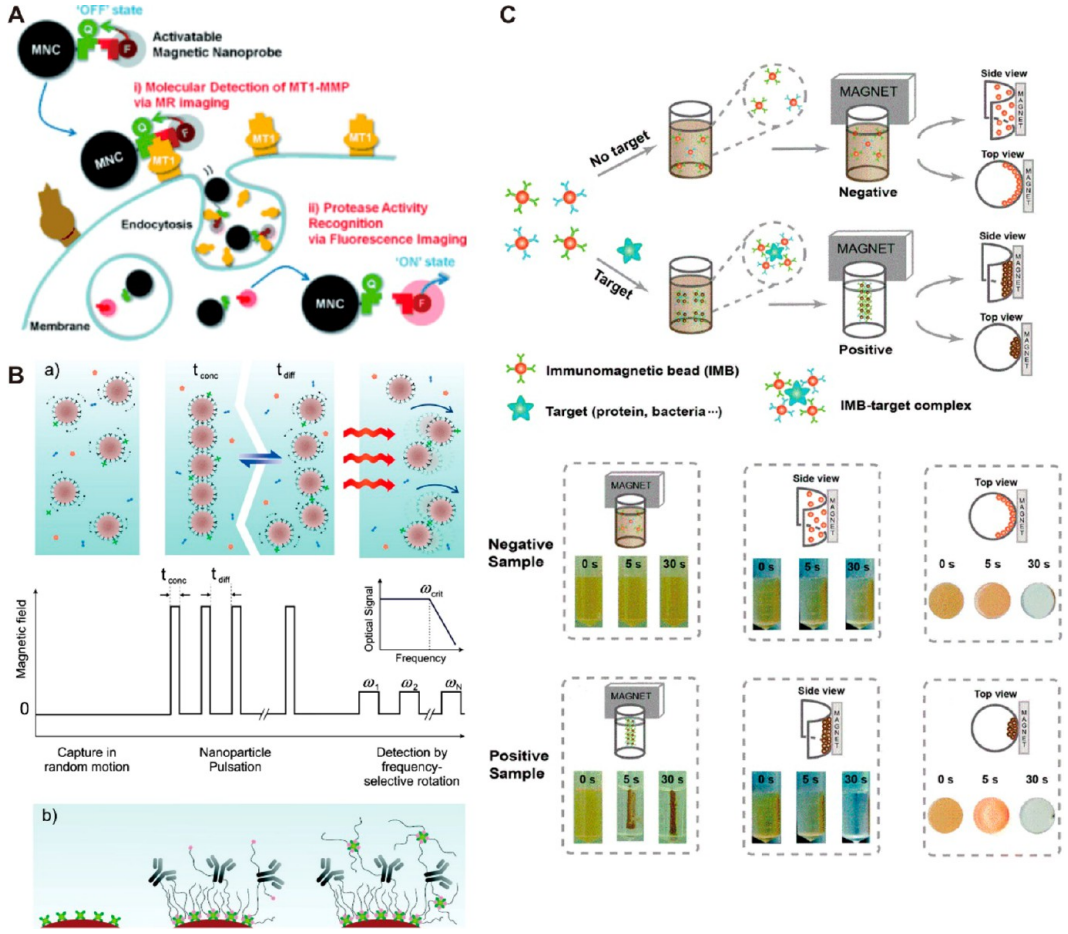

Figure 22. (A) Optomagnetic nanoprobes for detection of MT1-MMP anchored on invasive cancer cells by MR imaging and sensitive recognition of the proteolytic activity of MT1-MMP by fluorescence imaging. Reprinted with permission from ref 559. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA. (B) No-wash magnetic immunosensor for PSA. Reprinted with permission from ref 560. Copyright 2012 American Chemical Society. (C) Immunomagnetic aggregation sensor for naked-eye detection of protein biomarkers. Reprinted with permission from ref 564. Copyright 2016 The Royal Society of Chemistry.

plexing sensing platform for simultaneous detection of different protein biomarkers in a single assay was proposed by Ling and co-workers.[546] In addition, MRSw-based biosensing strategies have also been fabricated for monitoring tumor-related enzyme activities. There are two strategies for enzyme activity assay using MRSw. One is based on target-induced MNP aggregation similar to protein assay, which has been successfully applied for measuring enzyme activities of telomerase,[547,548] peroxidase[549] and caspase 3.[550] Yuan and colleagues[550] displayed an interesting MRSw-based no-wash biosensing system for caspase-3 by employing an enzyme-sensitive condensation reaction to control the aggregation of MNPs to decrease T_2 signal. Another strategy depends on a reverse switch, in which enzymatic cleavage of the substrates can cause the disassembly of preformed MNP clusters, further transducing the enzymatic activity into an enhanced T_2 signal. An outstanding contribution in this field was from Perez *et al.*,[551] who used a no-wash MRSw-based sensor to detect caspase-3 activity. A caspase-3-responsive MRSw nanoassembly was formed using the interaction of biotinylated peptide substrates and avidin-conjugated MNPs. With caspase-3, the MNP nanoclusters were disassembled by catalytic degradation at a selective site, causing a corresponding increase in T_2 relaxation time. Correspondingly, several similar MRSw-based no-wash sensors were widely employed to monitor DNA methylases,[552] trypsin,[553] lysozyme,[554] and MMP-2.[555] For larger biological objectives, these target molecules may serve as a scaffold to bind with functionalized MNPs, with the remaining free MNPs removed. The magnetic moment (change of $1/T_2$) is related to the number of MNPs bound, thus realizing the quantitative analysis of target analytes. These larger biological objectives are generally bacteria or cancer cells, *etc.* Lee *et al.*[556] reported a diagnostic MRSw-based no-wash sensor for the detection and molecular profiling of cancer cells by testing the change of T_2 in biological samples after the MNPs targeted cells. Subsequently, Haun and co-workers[557] further improved this sensor through bioorthogonal nanoparticle detection. This targeting sensing approach used 1,2,4,5-tetrazine (Tz) and trans-cyclooctene (TCO) as coupling agents to mediate the binding between cells and functionalized MNPs, where TCO-antibody conjugates could facilitate the coupling of Tz-coated MNPs (Figure 21A). Recently, Bamrungsap *et al.*[558] applied aptamers as an alternative to antibodies to achieve pattern-specific recognition of aptamer-modified MNPs and target cancer cells. Based on this strategy, no-wash magnetic nanosensor was constructed by recording the corresponding T_2 relaxation time change (Figure 21B).

Optomagnetic No-Wash Biosensors. Although the direct readout of magnetic signal is very interesting in magnetic sensing technology, optomagnetic readout has also been used

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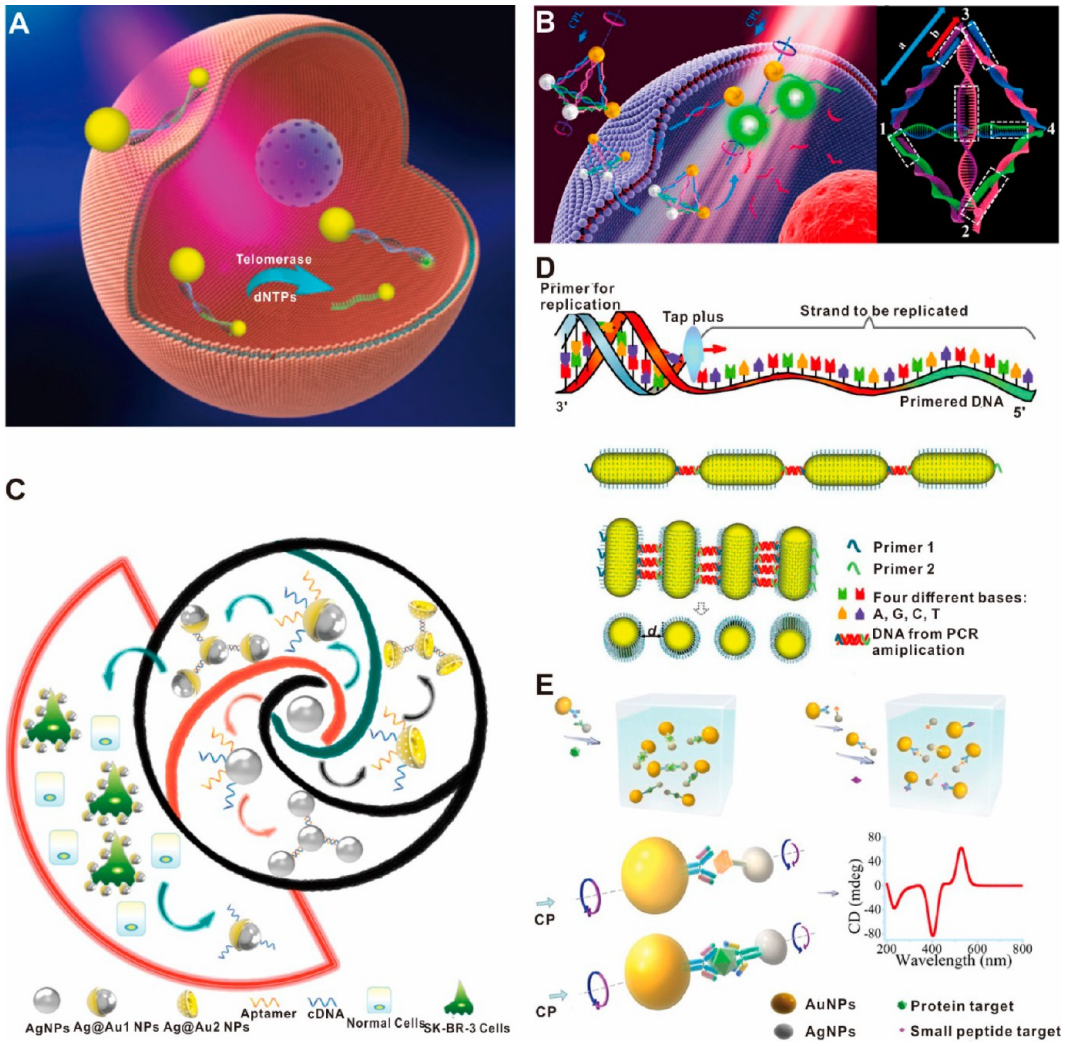

Figure 23. (A) Chiral gold heterodimers for intracellular telomerase quantification. Reprinted with permission from ref 572. Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA. (B) Au-UCNP pyramids for miRNA detection. Reprinted with permission from ref 574. Copyright 2016 American Chemical Society. (C) Propeller-like Ag@Au nanoparticle assemblies for chiroplasmonic analysis of cancer cells. Reprinted with permission from ref 577. Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA. (D) Chiral AuNR assemblies for DNA detection. Reprinted with permission from ref 578. Copyright 2013 Macmillan Publishers Limited. (E) Chiral immunosensor for PSA based on the assembly of nanoparticle heterodimers. Reprinted with permission from ref 579. Copyright 2013 American Chemical Society.

to design no-wash magnetic biosensors. As a proof of concept, Park *et al.*[559] prepared optomagnetic nanoprobes for the analysis of MMPs, where specific peptide substrate sequences with activatable fluorogenic labels and proteolytic ligand for MMP were synchronously conjugated onto the surface of magnetic nanocrystals as a targeting moiety and a fluorescence signal transducer (Figure 22A). In the presence of MMP, the quenched fluorescence of optomagnetic nanoprobes could be recovered, with additional MR imaging for cancer cells as a contrast agent. Soon after, Ranzoni *et al.*[560] reported on a no-wash optomagnetic immunosensor for PSA based on the interparticle binding of MNPs to form nanoclusters that could be sensitively measured by optical scattering through external magnetic rotation frequency (Figure 22B). Hereafter, a series of optomagnetic no-wash biosensors were described in the literature for determining C-reactive protein,[561] HER2,[562] and thrombin[563] *via* recording the corresponding optical scattering or absorption signal change. Very recently, an interesting wash-free magnetic immunosensor with naked-eye detection was introduced by Chen *et al.* (Figure 22C).[564] In the design, the aggregation of immunomagnetic MNPs was induced by target analytes to produce MNP nanoclusters on the side wall of a sample tube with an external magnetic field, thereby generating an obvious color change from yellow to brown, which could be easily identified with the naked eye. This sensor showed excellent detection performance for measuring AFP and CEA directly in spiked urine samples and patient serum samples.

OUTLOOK OF MAGNETIC NO-WASH BIOSENSORS

As stated earlier, many MRSw-based no-wash magnetic biosensors have been developed for sensitive detection of cancer markers *in vitro*. However, these biosensors rely mainly on target-induced aggregation or cluster formation of MNPs, which also suffers from the same disadvantages as those with AuNP aggregation-based colorimetric assays. Additionally, the simultaneous and multiplex detection of several biomarkers is limited for MRSw-based technologies. Moreover, another challenge for further application of MRSw-based magnetic no-wash biosensing involves the design of a portable miniaturized measurement device instead of traditional costly

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and complicated instruments. Compared with magnetic label-based assays, optomagnetic biosensors have been widely used as an alternative option for MNP-labeled target detection, which largely reduces the instrumentation cost without compromising analytical performance including sensitivity and selectivity, thus providing a huge potential for clinical implementation.

CHIRALITY-BASED NO-WASH BIOSENSORS

Circular dichroism (CD) spectroscopy is a frequently used tool for investigating the chiral properties of biomolecules. Chiral molecules without mirror symmetry or internal plane symmetry can present an optical CD signal originating from the interaction between the molecular rotation of chiral materials and left- and right-circularly polarized photons. Chiroptical methods have been widely employed to study the conformational changes of different biomolecules, like proteins and DNA.[565] The typically strong CD signals from these chiral biomolecules are observed in the ultraviolet and infrared range, owing to the electronic and vibrionic excitations of their chiral secondary structure, whereas a weak CD signal appears in the visible range. The weak detection signal of this chiroptical phenomena results in a low detection sensitivity at the microgram level, which largely limits their wide application in biosensing.[566] Previous study has proved that strong CD signals in the visible range could be produced through the collective Coulomb interaction of plasmonic dipoles in chiral NP assemblies.[567] The CD signal of assembled NPs was first reported by Alivisatos' group.[568] Later, a great deal of effort was devoted to the self-assembly and optical studies of various nanocomposites using individual noble metal NPs, QDs, UCPs, and biomolecules, *etc.*[569] These self-assembled nanostructures that exhibit strong optical chirality in the visible range can potentially be applied for exploring various chiroptical biosensing platforms. Currently, to construct the chiral nanoassemblies for different targets, molecular recognition-driven assembly of nanoparticles is one of the most commonly used methods, comprising nucleic acid hybridization and immunological recognition. Through the two molecular recognition strategies, different types of chiral nanostructures, containing dimers, pyramids, chains, as well as propeller-like assemblies, have been designed for the development of chiral biosensors. In recent years, a large number of chirality-based biosensing methods have been reported for cancer biomarkers, such as DNAs, miRNAs, enzyme proteins, as well as cells.[569] These chiral biosensors not only show unexpected sensitivity but also occur in solution. Zhao *et al.*[570] reported an ultrasensitive nanosensor for DNA detection in solution, where target DNA was used as a bridge to assemble two different sized metallic NPs to form heterodimers. Further deposition of gold and silver shells around the NP heterodimers was able to modulate the chiroplasmonic spectral bands for detection in the visible region. With target DNA, an increased CD signal was gained. Subsequently, a no-wash biosensor was developed for PSA by using DNA hybridization-driven Au@Ag nanorod dimers and CD probes coupled with a silver shell deposit.[571] When PSA was present, the preferred binding of the aptamer and target could trigger the disassembly of dimer, resulting in a decreased CD signal. Recently, Sun *et al.*[572] proposed a sensitive chiral sensor to monitor intracellular telomerase activity based on telomerase-triggered primer DNA elongation and chain substitution that can cause the disassociation of DNA-driven gold heterodimer, giving rise to a reduced CD signal, thereby facilitating the analysis of intracellular telomerase activity (Figure 23A). Besides dimer-based chiral sensors, pyramidal nanosensors based on DNA hybridization have been explored. Yan and colleagues[573] used four ssDNA and four identical AuNPs to form achiral pyramidal assemblies, and then target DNA hybridization was used to induce geometric change from the achiral pyramid to an asymmetric frame with strong chiroptical activity, thus producing a strong CD signal in the visible region. Thereafter, Li *et al.*[574] designed a chiroplasmonic nanopyramid for ultrasensitive measurement of miRNA *via* the self-assembly of AuNPs and UCPs by DNA hybridization (Figure 23B). With target miRNA, the specific hybridization of the recognition sequence and target led to the complete dissociation of the DNA frame, and the dissolution of the nanopyramid separated AuNPs and UCPs from each other. Therefore, the chiral activity of the pyramid disappeared, and the quenched luminescence of the UCPs originating from FRET was recovered. Consequently, a dual-mode signaling of CD and luminescence was recorded. Moreover, based on the degradation ability of endonuclease toward a DNA sequence, a pyramid-based chiral biosensor was fabricated for activity monitoring.[575] Propeller-like nanoassemblies have also been applied for developing chiral analysis approaches. Wu *et al.*[576] demonstrated a chiroptical sensing technology for target DNA assay through the use of propeller-like assemblies of AuNRs and UCPs as signal probes. In this case, the propeller-like nanoprobes were formed by DNA-driven self-assembly strategy, and this as-prepared sensor also exhibited the potential of dual-modal biosensing with chirality and luminescence. During the same year, Zhao *et al.*[577] reported on a similar propeller-like assembly for chiroplasmonic sensing of HER2-positive SK-BR-3 tumor cells by using DNA-driven self-assembly of Ag@Au core−shell NPs (Figure 23C). The specific aptamer with high affinity toward HER2 served as a recognition element for HER2-positive SK-BR-3 tumor cells, and the presence of HER2 can specifically bind with aptamer and then cause the dissociation of Ag@Au core−shell NP assemblies, leading to a decrease in CD signal, further realizing the quantitative analysis. Finally, chain assemblies of AuNRs with strong chiral signal were also established for target DNA analysis based on DNA-driven self-assembly method (Figure 23D).[578] Apart from DNA-driven self-assembly method, immunorecognition-driven self-assembly of nanoparticles has also been integrated with chiral biosensing platforms for cancer protein biomarkers. Wu *et al.*[579] proved a chiral immunosensor for the measurement of PSA in solution based on the formation of heterodimers of AuNPs and AgNPs along with the formation of sandwich immunocomplexes composed of target, antibody-labeled AuNPs, and antibody-conjugated AgNPs, where the resultant heterodimers showed a higher chirality, which could be used for the quantification of biological targets (Figure 23E). Although these reported chiral biosensing methods exhibit excellent detection performance in no-wash detection of cancer biomarkers, this sensing platform is still in the early development stage. The major challenge lies in the preparation of controllable and reproducible chair self-assembly of nanostructures. As a developing analytical method, further studies are necessary before clinical use, and more applications should be explored. In addition, another drawback of chirality-based methods is that complicated and expensive equipment are required to collect the detection signal.

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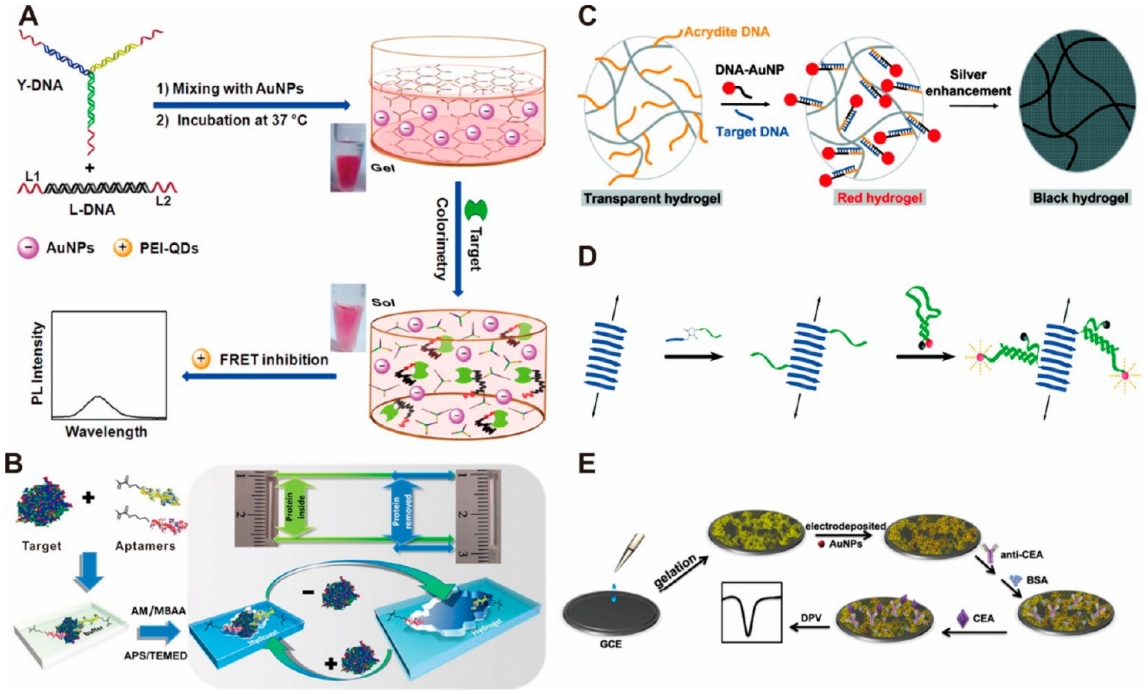

Figure 24. (A) Self-assembled DNA nanogel as a switchable material for colorimetric and fluorescent detection of thrombin. Reprinted with permission from ref 590. Copyright 2013 American Chemical Society. (B) Superaptamer-responsive nanogels for thrombin and PDGF. Reprinted with permission from ref 591. Copyright 2013 American Chemical Society. (C) DNA-functionalized nanogels and AuNPs for colorimetric DNA detection. Reprinted with permission from ref 592. Copyright 2010 American Chemical Society. (D) Fluorescence-based peptide nanogel DNA biosensor. Reprinted with permission from ref 606. Copyright 2016 The Royal Society of Chemistry. (E) AuNP-loaded polypyrrole nanogel-based electrochemical immunosensor for CEA. Reprinted with permission from ref 605. Copyright 2015 Nature Publishing Group.

NANOGEL-BASED NO-WASH BIOSENSORS

Nanogels are nanosized biomaterials with combined properties of hydrogels and nanomaterials, such as versatility, good biocompatibility, high storage stability and flexibility, low toxicity, as well as large specific surface area.[580−582] These excellent properties make nanogels a promising and potential platform for various biomedical applications including drug delivery systems, bioimaging and biotherapy, tissue engineering, and biosensing.[581,583] Among these developed nanogels, stimuli-responsive, smart, or intelligent nanogels have obtained considerable attention because of their special response ability to various external stimuli including pH, temperature, ionic strength, ultrasound irradiation, light, redox environment, electric charge, and antigen−antibody reaction.[580,583,584] Through the combination of these designed stimuli-responsive nanogels, a large number of nanogel-based biosensors have been reported for *in vitro* detection of various analytes, like small chemical molecules, heavy metal ions, and diseases-related biomarkers. Recently, several related reviews in this field have been presented elsewhere.[584−586] In this review, we mainly focus on the recent advances in nanogel-based biosensors for *in vitro* no-wash detection of cancer biomarkers. Compared with conventional nanogel-based biosensors, no-wash biosensors with nanogel are simpler, faster, cheaper, and more suitable for point-of-care testing. One of the main strategies for designing no-wash nanogel-based biosensors is based on analyte-responsive nanogels, which were fabricated through the incorporation of various specific functional biomolecules such as DNAs, aptamers, polypeptides, antibodies, and enzymes into the cross-linked nanogel networks during the synthesis.[581,585] The as-prepared stimuli-responsive nanogels can rapidly and specially respond to a variety of target analytes based on the special molecular recognition of biomolecules and an analyte of interest. With the special interaction between target analytes and biomolecules, the nanogel physical properties will produce a significant change such as gel degradation (gel−sol transition) or gel volume change,[587,588] thus enabling a direct detection of targets without any instruments. For example, Yang *et al.* first reported target-responsive nanogels that were fabricated by using two acrydite-modified oligonucleotides for targeting human thrombin based on the gel−sol transition under the assistance of aptamers.[589] Later, to enable the gel−sol conversion for biosensing, some nanomaterials including AuNPs and QDs as signal indicators have been integrated and amplified the response of nanogels to target molecules.[590] Zhang *et al.*[590] designed target-responsive DNA nanogel to develop a no-wash biosensor for the detection of thrombin by employing the DNA self-assembly of a Y-shaped DNA and an aptamer, where the special interaction of target thrombin and aptamer will lead to the gel degradation. The target-triggered gel−sol transition further causes the release of negatively charged AuNPs from the nanogels to bind the positively charged QDs, thereby resulting in a fluorescence quenching due to the presence of FRET between AuNPs and QDs to facilitate the sensitive quantitative analysis of thrombin (Figure 24A). Nevertheless, the main disadvantage of the gel−sol transition is that the nanogels cannot be easily regenerated after dissolution.[585] To overcome this problem, several intelligent gels with volume change have been reported for the fabrication of biosensor.[587] Bai *et al.*[591] first demonstrated superaptamer-responsive nanogels for the construction of no-wash biosensor to detect two cancer-related protein biomarkers of thrombin

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and PDGF on the basis of volume changing of nanogels. In this case, the target-bioimprinted aptamer nanogels were prepared through the strategy of superaptamer assembly to amplify the response to target proteins, where the volume shrinking of nanogels can be directly measured with the naked eye to enable ultrasensitive detection of targets (Figure 24B). Although these physical nanogel-based biosensors can be used to specifically target various analytes, they suffer from several limitations such as low sensitivity, slow response rates, as well as without any signal amplification.[585] Besides through sensing the physical changes of the nanogel, various strategies based on the usage of more sensitive signal transducers as an alternative of physical change of nanogel such as colorimetry,[592,593] fluorescence,[594−597] electrochemistry,[598,599] diffraction grating,[600] microfluidic distance,[601,602] and glucometer readout[603] have recently been introduced to improve the sensitivity of nanogel-based biosensors. These enhanced nanogel-based biosensors have been widely employed for *in vitro* detection of cancer-related biomarkers, including DNA,[592] miRNA,[594] enzyme,[604] and protein.[605] Baeissa *et al.*[592] first reported a nanogel-based colorimetric biosensor for sensitive detection of target by the combination of DNA-functionalized monolithic nanogel and AuNP-based colorimetric assay. In this study, two DNA molecules that are complementary with target DNA were conjugated into polyacrylamide nanogel and AuNP surface, respectively. In the presence of target DNA, the DNA-modified AuNPs are linked to the hydrogel surface to induce a red color on the gel surface for colorimetric detection of target DNA (Figure 24C). Recently, King *et al.*[606] described a self-assembling 3D peptide nanogel biosensor for the detection of target DNA by coupling with fluorescence MB probe to produce a fluorescent signal output, where the quenched fluorescence of MB probe can be recovered with the hybridization between the remaining single-stranded residues of the MB and sequence-specific oligonucleotide to form a more stable duplex for target DNA detection (Figure 24D). In combination with electrochemical signal output, Rong *et al.*[605] designed a 3D AuNP-loaded polypyrrole nanogel-modified glassy carbon electrode to fabricate a sensitive amperometric immunosensor for no-wash detection of CEA, where the network nanogel exhibits great surface area, high electron-transfer capability, as well as excellent conductivity (Figure 24E). Although nanogel-based no-wash biosensors play a key role in *in vitro* diagnostics of cancer, most of these developed biosensors are inadequate to satisfy the requirement of clinical applications because of the relatively low detection sensitivity, potential nonspecific interactions, poor multiplexing ability, as well as the complexity of real clinic samples.[581,585] Thus, further research should focus on overcoming these above obstacles.

CONCLUSION AND FUTURE PERSPECTIVES

Development of a simple, convenient, rapid, and sensitive biosensor platform for *in vitro* detection of cancer biomarkers is of great importance to improve clinical early diagnosis of cancer. Compared with heterogeneous biosensors, no-wash biosensors are a promising and exciting sensing platform for diagnosis because these methods are completed through simply mixing the target and recognition probes in solution without the need of separation or wash steps, which largely simplifies analytical procedures, shortens the total assay time, and minimizes human operation errors. Nevertheless, the analytical performance of this class of biosensing systems should be improved in terms of sensitivity, specificity, stability, reproducibility, and multiplexing detection capacity. The emergence of nanotechnology and nanomaterials provides a powerful driving force for the development of advanced no-wash biosensing systems. The excellent properties of nanomaterials exhibit great potential as effective signal amplification systems or straightforward signal transducers for the construction of various no-wash biosensors. This review provides a comprehensive overview of nanomaterial-mediated no-wash biosensors for the application of cancer biomarker detection *in vitro*. These no-wash detection strategies provide several strengths with respect to sensitivity, reliability, simplicity, low-cost, and user friendliness. Among these developed no-wash biosensing platforms, in our opinion, fluorescence and CL are the two most promising signal transduction approaches for fabricating no-wash biosensors and exhibit tremendous clinical translational value for point-of-care diagnostics owing to their relatively high sensitivity as well as easy and controllable signal readout. In particular, introduction of luminescent nanomaterials such as QDs as signal reporters effectively avoids the drawbacks of conventional organic luminophores including photobleaching, weak luminescence, small Stokes shift, short fluorescence lifetime, and autoluminescence and largely improves the detection performance of fluorescence and CL-based no-wash biosensors. On the other hand, other no-wash biosensors, such as colorimetric, electrochemical, DLS, SERS, magnetic, and chiral biosensors, exhibit relatively obvious disadvantages. For example, no-wash biosensors based on nanoparticle aggregation or self-assembly including colorimetric, DLS, SERS, chiral, as well as magnetic biosensors may experience nanoparticle autoaggregation or autodisassembly and are vulnerable to various environmental factors, easily causing false positive or negative results. In addition, the nanoparticle aggregation or assembly process for signal generation is dynamic and time dependent, which is unreliable for target quantification. Although colorimetric biosensors are simpler and more convenient for target qualitative detection by the naked eye, the background color of patient sample solution may interfere with the colored signal that influences the accuracy of detection. Electrochemical biosensors rely on the complicated modification of electrodes, show a poor selectivity, and are very sensitive to environmental changes such as pH and ion strength. Nevertheless, it is worth noting that a single-step and no-wash DLS immunosensor platform for target detection, named D2Dx (from diameter to diagnostics), is being commercialized by Nano Discovery Inc.

Although various no-wash biosensors have been designed for *in vitro* detection of cancer biomarkers, no specific biosensing method has been chosen as the "gold standard" for routine clinical application. One possible reason for this is that many of these reported no-wash biosensors remain at the proof-of-principle stage, and there is a significant difference in biosensor performance between the measurement of analyte in pure samples versus detection in complex biological samples such as serum, urine, and other body fluids. Large-scale clinical trials are required to prove the advantages of no-wash biosensors over the state of the art methods. Thus, there is still a long way to go before the widespread application of no-wash biosensors for effective clinical diagnosis. To explore the full potential of no-wash biosensors, in our opinion, future research directions should concentrate on the following aspects: First, due to the lack of washing or separation steps in no-wash biosensors, the greatest challenge is the background matrix interference of nontargets co-existing with the targets in patient samples, which can largely antagonize the analytical performance of detection

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methods. Recently, the usage of highly specific affinity recognition elements, such as antigen−antibody interaction, nucleic acid hybridization, or aptamer recognition has been used to improve detection sensitivity and specificity, but the overall enhancement effect is still suboptimal.

Second, further fundamental research should be completed prior to clinical implementation. For example, the detailed effects of different types of sample matrices including serum, plasma, urine, or other biological fluids on various no-wash biosensing platforms should be systematically investigated. In actual testing, clinical laboratorians should match the most suitable detection method for target analysis according to sample type to provide more objective and accurate diagnosis results.

Third, the multiplex detection of no-wash biosensors is a very complex problem due to the lack of effective separation methods for different targets and detection signals. Cross interference can result in inaccurate detection results. Although some progress has been made in multiplexed no-wash sensing for simultaneous detection of multiple cancer biomarkers by using nanomaterials, further improvement should be realized by introducing signal transducers with better differentiation capacity and less signal interference, integrating portable microfluidic devices and exploring better signal readout instruments.

Fourth, the advancements in nanoscience and nanotechnology confer significant advantages for no-wash biosensing systems. The detection sensitivity and multiplexing capacity are greatly improved with nanomaterials. However, these nanomaterial-based no-wash biosensors are limited by the challenges of controllable and scalable synthesis. Recently, the advent of microfluidic platform-based synthesis strategies has provided a promising approach for nanomaterial synthesis with high precision.

Fifth, surface modification of nanomaterials determines their stability and anti-interference ability against complex sample matrices, which is paramount for clinical application. Thus, investigation of effective surface modifiers is of great importance to fabricate nanomaterials with good stability and excellent matrix interference resistance ability.

Sixth, exploring some promising nanomaterials, signal transducers, and methods for analysis should be encouraged to spur the development of exciting no-wash biosensors for *in vitro* diagnosis of cancer. The cost of nanomaterials should also be considered, with additional consideration of the toxicity of such materials. With greater public interest in nanotechnology, it is expected that manufacturing processes will be streamlined over time, resulting in high-quality products and lower production costs.

In brief, great breakthroughs in fabricating no-wash biosensors for *in vitro* diagnosis of cancer will be realized in the near future under the multidisciplinary collaboration of a variety of experts including scientists, engineers, technical researchers, and clinicians.

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Notes

The authors declare no competing financial interest.

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