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Advances in single-molecule fluorescent nanosensors

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Abstract

Single-molecule detection represents the ultimate sensitivity in measurement science with the characteristics of simplicity, rapidity, low sample consumption, and high signal-to-noise ratio and has attracted considerable attentions in biosensor development. In recent years, a variety of functional nanomaterials with unique chemical, optical, mechanical, and electronic features have been synthesized. The integration of single-molecule detection with functional nanomaterials enables the construction of novel single-molecule fluorescent nanosensors with excellent performance. Herein, we review the advance in single-molecule fluorescent nanosensors constructed by novel nanomaterials including quantum dots, gold nanoparticles, upconversion nanoparticles, fluorescent conjugated polymer nanoparticles, nanosheets, and magnetic nanoparticles in the past decade (2011–2020), and discuss the strategies, features, and applications of single-molecule fluorescent nanosensors in the detection of microRNAs, DNAs, enzymes, proteins, viruses, and live cells. Moreover, we highlight the future direction and challenges in this area.

This article is categorized under:
Diagnostic Tools > Biosensing
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Diagnostic Tools > Diagnostic Nanodevices

KEYWORDS
fluorescence, nanomaterial, nanosensor, single-molecule detection

1 | INTRODUCTION

Selective detection of biomolecules with high sensitivity is highly desired in diverse research areas including disease diagnosis, food safety control, forensic analysis, and environmental monitoring (Hamburg & Collins, 2010; Lei & Ju, 2012; Ma et al., 2016). The traditional bioanalytical methods such as enzyme-linked immunosorbent assay

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(Lequin, 2005), western blotting (Kurien & Scofield, 2006), polymerase chain reaction (Erlich et al., 1991), and gel electrophoresis (Chrambach & Rodbard, 1971) are widely employed to detect biomolecules for a long time, but they suffer from obvious drawbacks of complicated procedures, long assay time, large sample consumption, and insufficient sensitivity for low-abundance analyst assay. Fortunately, with the development of fluorescence microscope and spectroscopy, the detection of single molecule via different fluorescence microscopies such as confocal fluorescence microscopy (Shelby & Chiu, 2003; Sun & Chiu, 2005), total internal reflection fluorescence microscopy (Loveland et al., 2012), and epifluorescence microscopy (Jiang et al., 2010) has been achieved. Superior to the conventional ensemble measurements, single-molecule detection can accurately measure individual fluorescent-labeled molecules and possesses distinct characteristics of low sample consumption, high sensitivity, high reliability, and rapid response time (Keller et al., 2002; Kozankiewicz & Orrit, 2014; Walt, 2013; Y. Wu et al., 2020), which makes them become the ideal detection platform for the construction of ultrasensitive biosensors (X. X. Han et al., 2009; Walt, 2013). For example, the integration of single-molecule detection with conventional pull-down assay can quantitatively detect proteins from 10 cells, while approximately 5000 cells are usually required in a typical western blot assay (Jain et al., 2011); the isothermal rolling-circle amplification-assisted single-molecule counting can detect single-copy gene and mutation in cytological samples (Lizardi et al., 1998), and the integration of single-molecule detection with fluorescent locked nucleic acid–DNA oligonucleotide probes can sensitively detect even 500 fM microRNA (Neely et al., 2006).

An emerging direction in single-molecule fluorescent biosensors development is the introduction of functional nanomaterials as sensing elements. Due to their different compositions, dimensions, sizes, and shapes, the nanoscaled materials exhibit unique chemical, optical, mechanical, and electronic properties, and they can be easily functionalized with different biomolecules through electrostatic binding, physical adsorption, ligand exchange, biorecognition, and covalent bonding for the construction of diverse nanosensors (Bellan et al., 2011; Ma, Zhang, & Zhang, 2020; Rosi & Mirkin, 2005; Sheehan & Whitman, 2005). Recently, a variety of nanomaterials have been employed for the development of novel single-molecule fluorescent nanosensors (Y. Han, Chen, et al., 2019; Y. Han, Ye, et al., 2019; B. Li et al., 2020; W. Li et al., 2016; X. Li et al., 2018; Ma, Ren, & Zhang, 2017; Ma, Wei, & Zhang, 2019; Pei et al., 2018; Z. Wu et al., 2019; J. Zhou et al., 2013). The use of functional nanomaterials including quantum dots, gold nanoparticles, upconversion nanoparticles, fluorescent conjugated polymer nanoparticles, nanosheets, and magnetic nanoparticles as fluorescent labels, quenchers, separators, and carriers has greatly improve the sensitivity, accuracy, and applications of single-molecule detection. Herein, we review the advances in the construction of single-molecule fluorescent nanosensors in the last 10 years (2011–2020). We briefly introduce the nanomaterials used in these nanosensors, and discuss the features, strategies, challenges, and applications of these single-molecule fluorescent nanosensors in the detection of microRNAs, DNAs, enzymes, proteins, viruses, and live cells.

2 | NANOMATERIALS USED IN SINGLE-MOLECULE FLUORESCENT NANOSENSORS

A variety of nanomaterials including gold nanoparticles (AuNPs), quantum dots (QDs), upconversion nanoparticles (UCNPs), fluorescent conjugated polymer nanoparticles (FCPNPs), manganese dioxide (MnO_2) nanosheets, and magnetic nanoparticles (MBs) are used for the construction of single-molecule fluorescent nanosensors. These nanomaterials can function as the fluorescent labels, fluorescent quenchers, separators, and carriers to facilitate the recognition, transduction, and amplification of target signals.

2.1 | Nanomaterials as the fluorescent labels

The fluorescent label is the core element of fluorescence detection and imaging system, and it can absorb light with certain wavelength and then efficiently be excited to emit detectable photons (Medintz et al., 2005). The QDs are small colloidal particles (Alivisatos, 1996; Hildebrandt et al., 2017; Wegner & Hildebrandt, 2015; J. Zhou, Yang, & Zhang, 2015), and are the most widely used fluorescent nanomaterials in single-molecule fluorescent biosensors. Especially, QDs are 20-fold brighter and 100-fold more photostable than the traditional organic dyes (Chan & Nie, 1998; Resch-Genger et al., 2008). Moreover, QDs have narrow, size-tuneable emission and broad absorption spectra, and multiple QDs can be simultaneously excited with a single wavelength (Medintz et al., 2005), facilitating the simultaneous detection of various multiple biomolecules (M. Han et al., 2001). These brilliant properties make QDs become attractive fluorescent

nanomaterials for biomolecule detection and imaging (Coropceanu & Bawendi, 2014; Klimov et al., 2000; Lemon et al., 2015; Michalet et al., 2005; Yin & Alivisatos, 2005).

The FCPNPs are organic macromolecules with the characteristic of extended π-conjugation (Baier et al., 2009). The FCPNPs possess abundant π-electrons and the semiconductor-like properties, and they can emit strong fluorescence emission (Ng & Zheng, 2015). Moreover, the FCPNPs have distinct advantages of small size, facile preparation, bright fluorescence emission, good biocompatibility, low cytotoxicity and exceptional photostability, and they are easily conjugated with multiple ligands through either functional molecules modification or polymers copolymerization, facilitating biological sensing, drug delivery, cell imaging, and single-particle tracking (Childress et al., 2012; Dmitriev et al., 2015; Feng et al., 2010; Ke et al., 2017; C. F. Wu & Chiu, 2013; Yu et al., 2009).

The UCNPs consists of a rare earth element crystalline host (such as $NaYF_4$ and $NaGdF_4$) doped with lanthanide ion (e.g., Yb^{3+}, Tm^{3+}, and Er^{3+}) (Boyer et al., 2007). The lanthanide ion in UCNPs has 4f inner shell electron configuration whose intra-4f and 4f–5d electron transitions can induce the emission of fluorescence (Dong et al., 2015). Different from the QDs and FNPs, the UCNPs possess an anti-Stokes shift which enables the absorption of a lower energy photon and the emission of a higher energy photon. The UCNPs can be excited by a near-infrared (NIR) light to minimize the autofluorescence from cells and tissues (Haase & Schäfer, 2011; Rodríguez-Sevilla et al., 2015). Moreover, the tuning of UCNPs emission wavelength can be obtained by adjusting the amounts and types of lanthanide ion dopants, making them suitable for multiplexed assays (S. Cao et al., 2018; Kurt et al., 2016).

In the single-molecule fluorescent nanosensors, these fluorescent nanomaterials can specifically label the target biomolecule and emit strong fluorescent signal which can be detected via single-molecule imaging (X. Li et al., 2018; Pei et al., 2018; Zhou et al., 2013).

2.2 | Nanomaterials as the fluorescent quenchers

The fluorescent quencher can decrease the fluorescence intensity of certain fluorescent label through various mechanisms such as energy transfer, static quenching, and collisional quenching, facilitating the homogenous fluorescent detection through target-induced quenching or dequenching process (Anger et al., 2006). The AuNPs have been widely applied to construct various nanosensors (Boisselier & Astruc, 2009; Giljohann et al., 2010; W. Zhou, Gao, et al., 2015). The AuNPs can be easily synthesized and stably stored, and they possess unique chemical and physical properties such as excellent biocompatibility, high surface-to-volume ratio, distance- and size-dependent color changes, and unique chiroptical characteristics (Daniel & Astruc, 2004). Moreover, the AuNPs can be easily modified with organic/biological ligands through highly stable Au-S bond, facilitating the detection of various biomolecules (Zayats et al., 2005). Because AuNPs have broad ultraviolet visible absorption bands and high extinction coefficients, and may function as efficient fluorescence quencher (Oh et al., 2005; Sapsford et al., 2006), which makes them become popular fluorescence quenchers in the construction of fluorescent biosensors (Peltomaa et al., 2018; Y. Wang et al., 2014; Y. Yang et al., 2015). MnO_2 nanosheet is a kind of two-dimensional nanomaterial with the features of high light absorption capability and large surface area (H. Wang, Zhang, et al., 2015; Yan et al., 2018), and its broad absorption spectrum makes them become efficient fluorescence quenchers besides AuNPs (Deng et al., 2011; Zhao et al., 2014). In single-molecule fluorescent nanosensor, the AuNP and MnO_2 nanosheet can quench the fluorescent labels. When target biomolecule is present, it causes the separation of fluorescent labels from the quencher, leading to the recovery of fluorescent signal, which can be quantified by single-molecule detection (Y. Han, Chen, et al., 2019; Y. Han, Ye, et al., 2019; B. Li et al., 2020; X. Li et al., 2018).

2.3 | Nanomaterials as the separators and carriers

The separator indicates the substance that can separate interest target from the unwanted other components in a solution (Tekin & Gijs, 2013), and the carrier refers to the substance that can load multiple sensing probes or elements on its surface (Chrambach & Rodbard, 1971). The MBs with magnetic elements (e.g., cobalt, nickel, and iron) can be manipulated by using a magnetic field (Liwei et al., 2015). The MBs has unique magnetic features (especially the superparamagnetism), which allows for rapid and highly efficient separation and concentration of targets under a magnetic field (Tekin & Gijs, 2013). Moreover, the MB are essential parts of bar-code assay, in which the MBs serve as the probe carriers to convert a small number of targets into a large number of bar-code strands, achieving efficient amplification

of target signal (Stoeva et al., 2006; Thaxton et al., 2005). In single-molecule fluorescent nanosensor, the MBs can function as the separators to separate and concentrate signal probes (Zhou et al., 2013), viruses (Z. Wu et al., 2019), live cells (Ma, Wei, & Zhang, 2019), and they can serve as the carriers to amplify the protein signal as well (Chrambach & Rodbard, 1971).

3 | BIOSENSING APPLICATIONS

Single-molecule fluorescent nanosensors have been employed to detect various targets including nucleic acids (including DNAs and microRNAs), proteins, enzymes (e.g., DNA modifying enzymes and protein modifying enzymes), viruses, and live cells.

3.1 | Detection of DNAs and DNA modifications

DNAs are universal genetic materials in living organisms (Duzdevich et al., 2014). Sequence-specific DNA detection is essential to disease diagnosis, gene analysis, food safety control, forensic analysis, environmental monitoring, and drug discovery (Bell, 2004; Ma, Ren, & Zhang, 2017; Sassolas et al., 2008). Wang et al. constructed a QD-based single-molecule fluorescent nanosensor to sensitively detect DNA (Z. Wang et al., 2012). They designed a DNA tetrahedron structure by self-assembling four different DNA strands, with one pendant probe and three amino groups being positioned at its four vertices, respectively (Figure 1(a)). The DNA tetrahedron is immobilized onto a glass slide via the epoxy–amino reaction. The presence of target DNA results in the formation of detection DNA/target DNA/pendant probe sandwiched structure. Because the detection DNA is labeled with biotin, the streptavidin-coated QDs will bind the sandwiched structure through streptavidin–biotin interaction, enabling the capture of QDs on glass slide surface. Lastly, the immobilized QDs can be measured by single-molecule detection. In this assay, the introduction of DNA tetrahedron structure for target DNA recognition can reduce the nonspecific adsorption, efficiently minimizing the background signal. The detection limit can reach 1 fM. Moreover, this single-molecule fluorescent nanosensor exhibits good assay performance even in 20% serum samples.

Simultaneous measurement of multiple DNA targets can greatly enhance the assay throughput and reduce the analysis time. Zhou et al. constructed a QD-based single-molecule fluorescent nanosensor to simultaneously detect multiple DNAs (Zhou et al., 2013). As shown in Figure 1(b), the liposomes/QDs (L/QD) complexes are prepared in liposomes through the encapsulation of QDs, with hundreds of QD particles in one L/QD complex. Subsequently, reporter probe is conjugated to the L/QD complex, and capture probe is conjugated to the magnetic bead. The target DNA can simultaneously bind capture probe and reporter probe to form a L/QD/target DNA/magnetic bead complex. After the magnetic separation, the separated L/QD complexes can be disrupted by chloroform to release the QDs. The free QDs can be easily counted using single-particle detection. The proposed nanosensor can simultaneously detect HIV-1 and HIV-2 DNAs using two different color L/QD complexes. The sensitivity can reach attomolar.

Pei et al. developed a fluorescent polymer nanoparticle (FNP)-based single-molecule fluorescent nanosensor to simultaneously detect three single-nucleotide variants (SNVs) (Pei et al., 2018). They conjugated magnetic bead with capture DNA to obtain the capture DNA-MB, and conjugated the FNP with signaling DNA to obtain the signaling DNA-FNP (Figure 1(c)). In the presence of target SNV, the capture DNA-MB/SNV/signaling DNA-FNP sandwich-structured complex is obtained, which can be magnetic separated from the unbound FNP. After NaOH treatment, the FNP is released from the complex and is subsequently subjected to single-molecule counting. In this assay, a masking reagent (MH) with a hairpin structure, which is perfectly complementary to wild-type (WT) DNA sequence, is added to prevent the nonspecific binding. By using FNPs with three different colors, this single-molecule fluorescent nanosensor can simultaneously detect three SNVs in Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene with the discrimination factor of 545 and the sensitivity of 0.05–0.1% abundance, and it can measure genomic DNA SNV in cancer cells with the sensitivity of 0.05% abundance.

DNA methylation is an important epigenetic modification (Robertson, 2005), and the aberrant methylation is associated with a variety of human genetic diseases (Laird, 2003; Schübeler, 2015). Consequently, DNA methylation can serve as a biomarker (Q. Zhang et al., 2021). Wang et al. developed a tricyclic ligase chain reaction (LCR)-mediated QD-based Förster resonance energy transfer (FRET) nanosensor to detect DNA methylation at both non-CpG and CpG sites (Z.-Y. Wang et al., 2018). FRET is a physical process that the energy is transferred from a high-energy donor fluorophore to a

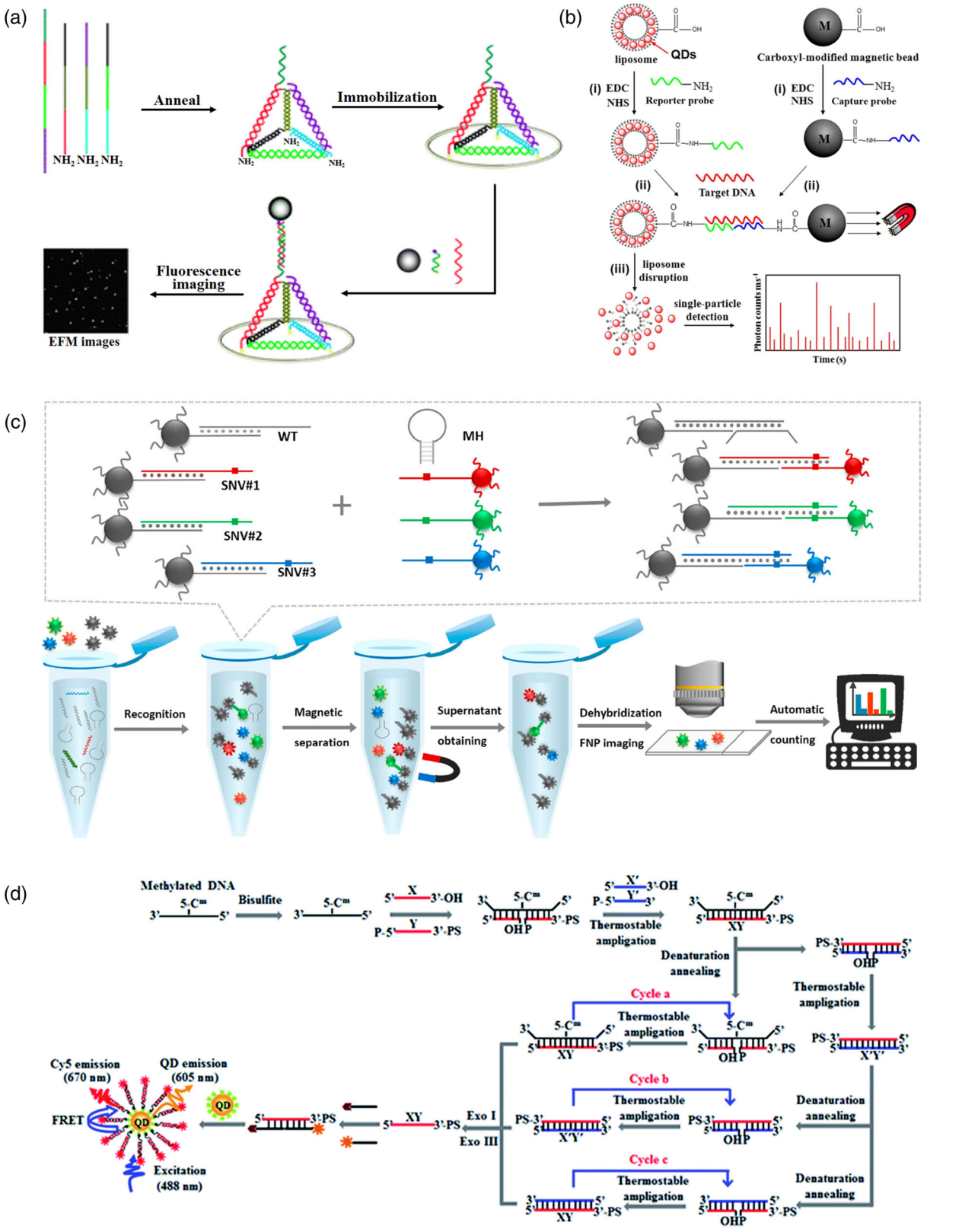

FIGURE 1 (a) QD-based single-molecule fluorescent nanosensor for single DNA target detection. Reprinted with permission from (Z. Wang et al., 2012). Copyright 2012 The Royal Society of Chemistry. (b) QD-based and MB-based single-molecule fluorescent nanosensor for multiplexed detection of two DNA targets. Reprinted with permission from Zhou et al. (2013). Copyright 2013 American Chemical Society. (c) FNP-based and MB-based single-molecule fluorescent nanosensor for multiplexed detection of three DNA targets. Reprinted with permission from Pei et al. (2018). Copyright 2018 American Chemical Society. (d) QD-based single-molecule fluorescent nanosensor for DNA methylation detection. Reprinted with permission from Z.-Y. Wang et al. (2018). Copyright 2018 The Royal Society of Chemistry

lower energy acceptor fluorophore in a distance-dependent manner, which enables the homogenous target detection without any separation steps (Marx, 2017). In this nanosensor, the bisulfite treatment cannot change the methylated DNA. The hybridization of methylated DNA with probes X and Y results in the formation of the ligated XY products with the assistance of DNA ligase (Figure 1(d)). With the XY strand as the template, probes X′ and Y′ can be ligated to obtain the X′Y′ strand. Subsequently, with the X′Y′ strand as the template, probes X and Y can be ligated to obtain the XY strand. The cyclic ligase chain reaction results in the generation of abundant XY strands which are resistant to exonucleases I and III due to the phosphonothioate modification at 3′ end of probe Y. The hybridization of XY strand with reporter and capture probes leads to the formation of sandwich hybrids which can assemble on the surface of 605QD to induce efficient FRET between Cy5 and 605QD. The FRET signal can be measured by single-molecule detection. While in the presence of unmethylated DNA, bisulfite treatment enables the conversion of cytosine to uracil. As a result, the ligase chain reaction cannot be triggered, and no FRET occurs. This nanosensor can achieve a detection limit of 1.0 aM and even single 5-methylcytosinee resolution, and it can detect DNA methylation in one single human lung cancer cell.

3.2 | Detection of microRNAs

MicroRNAs are small, endogenous noncoding RNAs (Bartel, 2004) with the function of post-transcriptional modulators (Filipowicz et al., 2008). MicroRNAs play essential roles in various cellular processes such as cell apoptosis, metabolism, and proliferation (Chen et al., 2006; Welch et al., 2007; Xu et al., 2003), while the alteration of microRNA expression levels is closely linked to various human diseases including cancers (Lin & Gregory, 2015). The detection of microRNAs remains challenging due to their small size, low abundance, and high sequence similarity among family members (Dong et al., 2013). Zhang et al. constructed a QD-based single-molecule fluorescent nanosensor to specifically detect let-7a using isothermal exponential amplification reaction (EXPAR) (Zhang & Zhang, 2011). The EXPAR is characterized by rapid amplification kinetics, isothermal condition, and high amplification efficiency (Ma, Yang, & Zhang, 2014). As shown in Figure 2(a), target microRNA can hybridize with the template X′-X′ and is subsequently extended by Vent (exo-) DNA polymerase, generating a dsDNA with a Nt.BstNBI recognition site. The upper strand of DNA duplex can be cleaved by Nt.BstNBI to produce short ssDNA X_0. Because the X_0 has identical sequence with that of target microRNA, the X_0 can bind a new X′-X′ template to initiate next round of polymerization and nicking reaction. As a result, an exponential reaction is achieved to produce large amounts of X_0 strand. The subsequent binding of X_0 with the second template X′-Y′ can trigger a linear amplification with the assistance of Vent (exo-) DNA polymerase and Nt.BstNBI, producing abundant reporter DNA strand Y. The Y strand can be sandwiched by a Cy5-labeled reporter probe and a biotinylated capture probe to form the biotin–DNA–Cy5 complex whose assembling on the 605QD surface can cause efficient FRET between Cy5 and 605QD. The resultant Cy5 signal can be easily detected by microfluidic flow-based single-molecule counting. This nanosensor possesses near-zero background and its detection limit can reach 0.1 aM. Moreover, it has the capability of discriminating even single-nucleotide difference.

To avoid laborious enzyme-assisted nucleic acid amplification steps involved in above nanosensors, Zhang et al. constructed a QD-based single-molecule fluorescent nanosensor to detect microRNA based on enzyme-free toehold-mediated strand displacement cascade (Ma, Zhang, & Zhang, 2019). As shown in Figure 2(b), target microRNAs can bind the toehold domain a* of the Cy5-labeled signal probe and trigger the branch migration reaction to expose the single-stranded toehold domain c. The exposed domain c can subsequently bind the biotinylated capture probe to trigger the branch migration reaction, generating a signal probe/capture probe duplex and meanwhile displacing microRNA from signal probe. The binding of the released microRNAs with other signal probes can initiate cyclical strand displacement reaction, generating abundant biotin/Cy5 dual-labeled DNA duplexes. The assembling of the obtained duplexes on the streptavidin-coated 605QD can induce efficient FRET which can be measured by single-molecule detection. In this nanosensor, microRNA can act as the catalyzer for signal amplification, without the requirement of any protein enzymes for target signal amplification. Moreover, this nanosensor can sensitively detect 1 fM miR-21 and possesses single-base resolution. It can be successfully applied to detect circulating microRNA from lung patients' blood and endogenous microRNA in cancer cells.

Liu et al. took advantage of enzyme-free toehold-mediated strand displacement cascade strategy to develop a AuNP-based single-molecule fluorescent nanosensor to detect microRNA (B. Li et al., 2020). The H1-AuNP probe is obtained by assembling Cy5-labeled H1 probe on the AuNP surfaces, with the Cy5 signal being efficiently quenched by AuNP (Figure 2(c)). The toehold domain 1 of H1 can hybridize with target microRNA to trigger a branch migration reaction, leading to the exposure of toehold domain 3* of H1. The Cy3-modified H2 probe can subsequently hybridize with

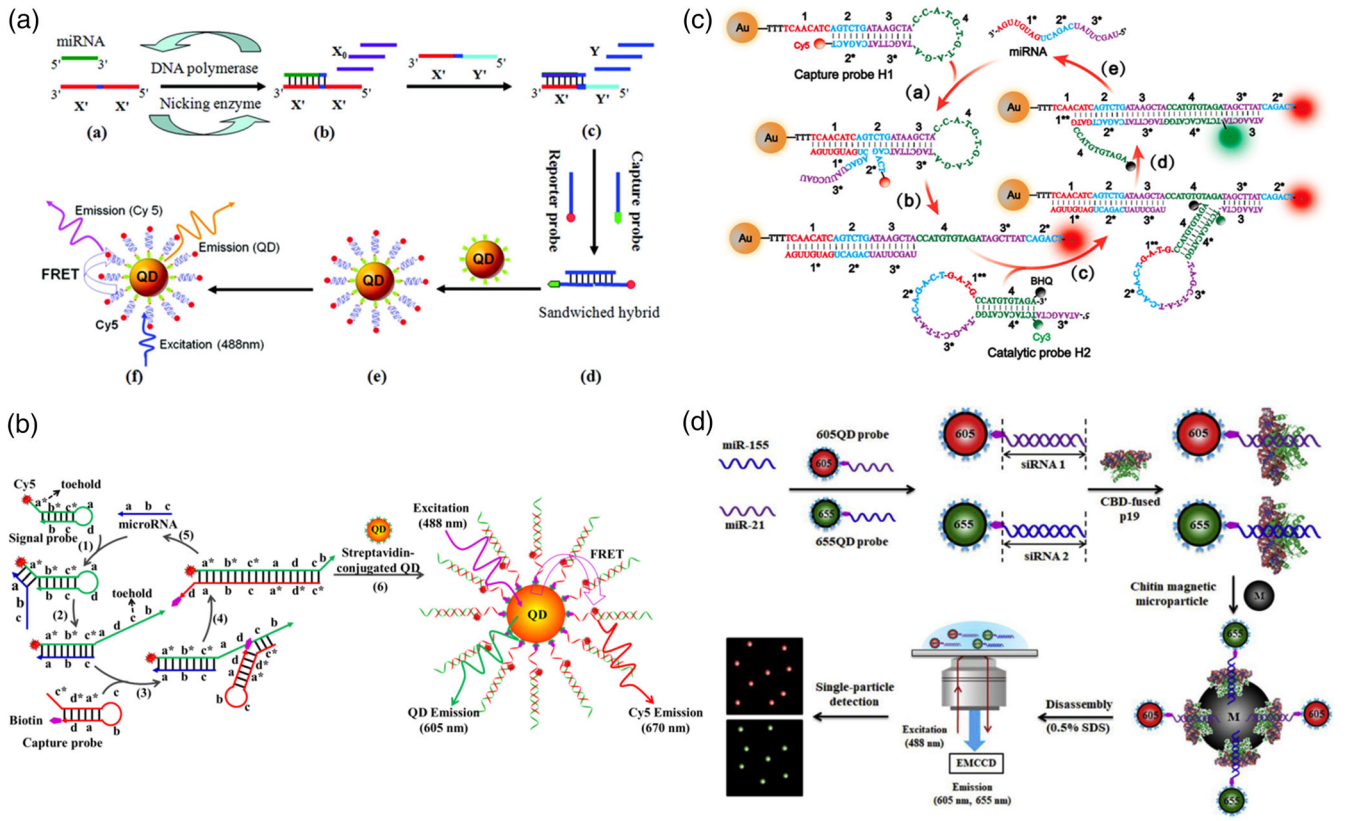

FIGURE 2 (a) QD-based single-molecule fluorescent nanosensor for microRNA assay using two-stage EXPAR. Reprinted with permission from Zhang and Zhang (2011). Copyright 2011 American Chemical Society. (b) QD-based single-molecule fluorescent nanosensor for microRNA detection using toehold-mediated strand displacement cascade. Reprinted with permission from Ma, Zhang, and Zhang (2019). Copyright 2019 American Chemical Society. (c) AuNP-based single-molecule fluorescent nanosensor for microRNA detection using toehold-mediated strand displacement cascade. Reprinted with permission from B. Li et al. (2020). Copyright 2020 American Chemical Society. (d) QD-based single-molecule fluorescent nanosensor for simultaneous measurement of multiple microRNAs. Reprinted with permission from Ma, Jiang, and Zhang (2020). Copyright 2020 Elsevier B. V.

domain 3* of H1 to initiate another branch migration reaction, resulting in the formation of H1/H2 duplex and the release of microRNA. The free microRNAs can induce cycles of toehold-mediated strand displacement reaction, generating abundant H1/H2 duplexes. As a result, the separation of Cy3 and Cy5 moieties from the AuNP leads to the recovery of Cy3 and Cy5 signals. The colocalization of Cy5 and Cy3 signals can be detected through single-molecule imaging. In this nanosensor, the dual-color colocalization detection strategy greatly improves the detection accuracy and reduces the false-positive signal, and the integration of single-molecule detection with toehold-mediated strand displacement cascade can achieve a detection limit of 1 fM for miR-21. This nanosensor can be further used for real-time imaging of miR-21 in living cells.

The simultaneous measurement of multiple microRNAs is essential to clinical diagnosis, because one disease is usually linked to the deregulation of multiple microRNAs (Porkka et al., 2007). Zhang et al. developed a siRNA-directed self-assembled QDs nanosensor for simultaneous single-molecule detection of multiple microRNAs (Ma, Jiang, & Zhang, 2020). As shown in Figure 2(d), the QD nanoprobes are obtained by the conjugation of different-color QDs (i.e., 605QD and 655QD) with two single-stranded RNAs, which can sequence-specifically bind targets miR-155 and miR-21 to obtain the 605QD-siRNA 1 and the 655QD-siRNA 2, respectively. The obtained QDs-siRNAs can react with p19 and subsequently assemble on the chitin-functionalized magnetic microparticle via specific chitin-binding domain (CBD)-chitin interaction, generating the QDs-siRNAs-p19-MMP bioconjugates. Sodium dodecyl sulfate (SDS) can denature p19 to release the QDs probes from the magnetic microparticle, and the released QDs can be measured by single-particle counting. This nanosensor can simultaneously detect multiple microRNAs without the need of any organic dyes and enzyme/chemical reactions. Moreover, this nanosensor can be used to simultaneously measure multiple circulating microRNAs in clinical samples.

3.3 | Detection of proteins

Proteins are macromolecules involved in important biological functions such as replicating genomic information, regulating transcription, signaling, providing structure, and transporting molecules. Some proteins are dysregulated and differentially expressed in disease and can serve as the biomarkers and drug targets. Therefore, methods for proteins detection are essential to the characterization of these functions (Cohen & Walt, 2019). Protein biomarkers often have following two properties: (a) they cannot be "amplified" as nucleic acids; (b) they are very sensitive to ambient environment (e.g., salt concentration, temperature, and pH). These properties make it difficult to detect low-abundant proteins. In addition, many crude samples are complex, for example, the blood contains various proteins with high abundance. Accurate identification of specific proteins in such a high background of interfering proteins is a challenging task. The introduction of single-molecule fluorescent nanosensor make it possible to sensitively and selectively detect proteins (Y. Zhang et al., 2013).

Prostate-specific antigen (PSA) can be used as a key oncological biomarker for prostate cancer diagnosis (Lilja et al., 1987). Xiao et al. developed a UCNPs and AUNP-based single-molecule fluorescent nanosensor for PSA assay (X. Li et al., 2018). The anti-PSA antibody Ab_1-conjugated UCNPs (UCNPs-Ab_1) serves as the luminescence energy donor, and the anti-PSA antibody Ab_2-conjugated AUNPs (AUNPs-Ab_2) serves as the luminescence energy acceptor (Figure 3(a)). The binding of target PSA with both Ab_1 and Ab_2 induces the close of donor to acceptor and consequently the dosage-dependent quenching of fluorescence as a result of luminescence resonance energy transfer. In the absence of PSA, the UCNPs-Ab_1 and AUNPs-Ab_2 will separate from each other, and no luminescence resonance energy transfer occurs. The UCNPs fluorescence signal can be detected by single-particle counting in a flow chamber. This nanosensor exhibits excellent specificity in serum samples, and it can achieve a detection limit of 1.0 pM and a dynamic range of 0–500 pM. Nevertheless, the above nanosensor detects the PSA in signal-off manner, which complicates the data analysis and may cause the false positive.

Farka et al. developed a UCNPs-based single-molecule immunosorbent nanosensor to detect PSA in a signal-on manner (Farka et al., 2017). As shown in Figure 3(b), the UCNPs-labeled antibody serves as the luminescent reporter, and it can be captured by 96-well microtiter plate through sandwich immunoassay which involves the immobilization of anti-PSA capture antibody on the plate, the binding of target PSA with capture antibody, and the recognition of captured PSA by UCNPs-labeled antibody. The immobilized UCNPs signal can subsequently be detected by inverted wide-field epiluminescence microscope. This nanosensor can achieve a detection limit of 42 fM and a dynamic range of 100 pg/mL to 10 ng/mL in 25% blood serum.

Cytokines are widely participate in the signaling pathways involved in tumor cell survival, invasion, and metastasis (Harari et al., 2004; Popa et al., 2007), and they may function as the important biomarkers in clinical diagnosis (Rifai et al., 2006). Wang et al. constructed a single-molecule fluorescent nanosensor to simultaneously detect tumor necrosis factor-α (TNF-α) and cytokines interferon-γ (IFN-γ) based on magnetic nanobead-assisted dual bar-code strategy (W. Li et al., 2016). As shown in Figure 3(c), the antibodies of TNF-α and IFN-γ are immobilized on the glass substrate, and the streptavidin-magnetic nanobeads are labeled with both second antibodies and single-stranded DNAs. The sandwich-type immunocomplexes can be formed on the substrate through specific antibody–antigen reaction. After washing the unbound nanobeads, the single-stranded DNAs on the remained nanobeads can serve as the triggers to induce multibranched hybridization chain reaction (mHCR) with two sets of fluorescent probes, generating the amplified fluorescence signals which can be detected by single-molecule counting. Due to the introduction of single-molecule detection and bar-code-based amplification strategy, this nanosensor can simultaneously quantify two targets with a detection limit of 5 fM for both IFN-γ and TNF-α, and it performs well in human serum samples.

3.4 | Detection of DNA modifying enzymes

DNA methyltransferases can induce DNA methylation at the specific location and establish special DNA methylation pattern (Z.-M. Zhang et al., 2018). The dysregulation of DNA methyltransferase activity has been closely linked to a variety of human diseases (Cai et al., 2017). Zhang et al. constructed a QD-based single-molecule fluorescent nanosensor to detect DNA adenine methyltransferase (Dam) activity (Ma, Liu, et al., 2017). As shown in Figure 4(a), a biotinylated hairpin DNA is designed as the detection probe. This detection probe is labeled with Cy5 and BHQ2 at the opposite strand of the stem and contains the target Dam recognition sequence 5′-GATC-3 in the stem. The assembling of detection probes on the QD surface through streptavidin–biotin interaction can induce FRET between 605QD and

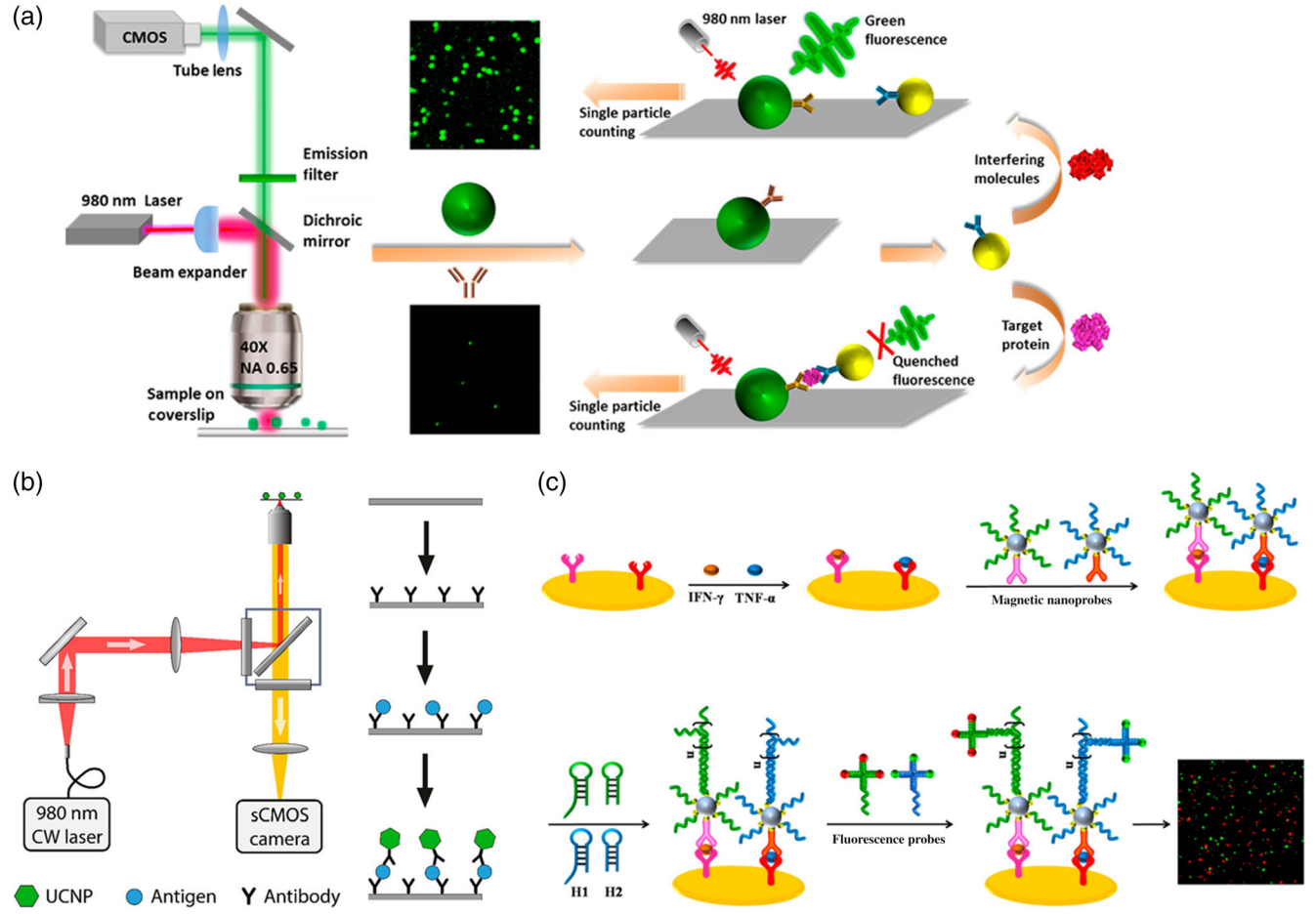

FIGURE 3 (a) UCNP and AUNP-based single-molecule fluorescent nanosensor for PSA detection. Reprinted with permission from X. Li et al. (2018). Copyright 2018 American Chemical Society. (b) UCNPs-based single-molecule fluorescent nanosensor for PSA detection. Reprinted with permission from Farka et al. (2017). Copyright 2017 American Chemical Society. (c) Magnetic bead-based single-molecule fluorescent nanosensor for cytokine detection. Reprinted with permission from W. Li et al. (2016). Copyright 2015 American Chemical Society

Cy5 and consequently the emission of Cy5. However, the nearby BHQ2 can efficiently quench the Cy5 fluorescence. The presence of target Dam induces the methylation of detection probes and their subsequent cleavage by DpnI restriction enzyme causes the separation of BHQ2 from the detection probes. As a result, Cy5 fluorescence is recovered. The proposed nanosensor is very simple and sensitive with a detection limit of 2 U/μl. It can be used to screen Dam inhibitor and perform well in human serums. With the introduction of terminal deoxyribonucleotidyl transferase (TdT)-catalyzed signal amplification, the QD-based single-molecule fluorescent nanosensor can sensitively detect DNA methyltransferase M.SssI with a detection limit of 2.1×10^{-7} U/μL (Hu et al., 2020).

The above nanosensors require specific restriction endonuclease for target DNA methyltransferase signal recognition, which is relatively complicated and expensive. Alternatively, Liu et al. introduced a QD-based single-molecule fluorescent nanosensor to directly detect M.SssI using antibody for target recognition (H. Zhang, Zhang, Yao, et al., 2019). As shown in Figure 4(b), a double-strand DNA (dsDNA) is designed as a detection probe and it contains the M.SssI recognition sites. The detection probes are immobilized on the coverslip surface. In the presence of M.SssI, the detection probe is methylated and subsequently bind the biotinylated 5mC Ab to form the dsDNA–5mC Ab complex which can specifically bind the streptavidin (SA)-labeled quantum dots (QS 585). The immobilized QS 585 on the coverslip surface can be quantified by single-molecule imaging. This nanosensor can directly detect methylated DNA sites without the involvement of either exonuclease or restriction endonuclease. Moreover, this nanosensor can be employed to screen the DNA MTase inhibitors.

DNA glycosylases are key DNA repairing enzymes in base excision repair (BER) pathway (Drohat & Coey, 2016; Stivers & Jiang, 2003). Aberrant DNA glycosylases activity can interfere the DNA repairing processes and induces

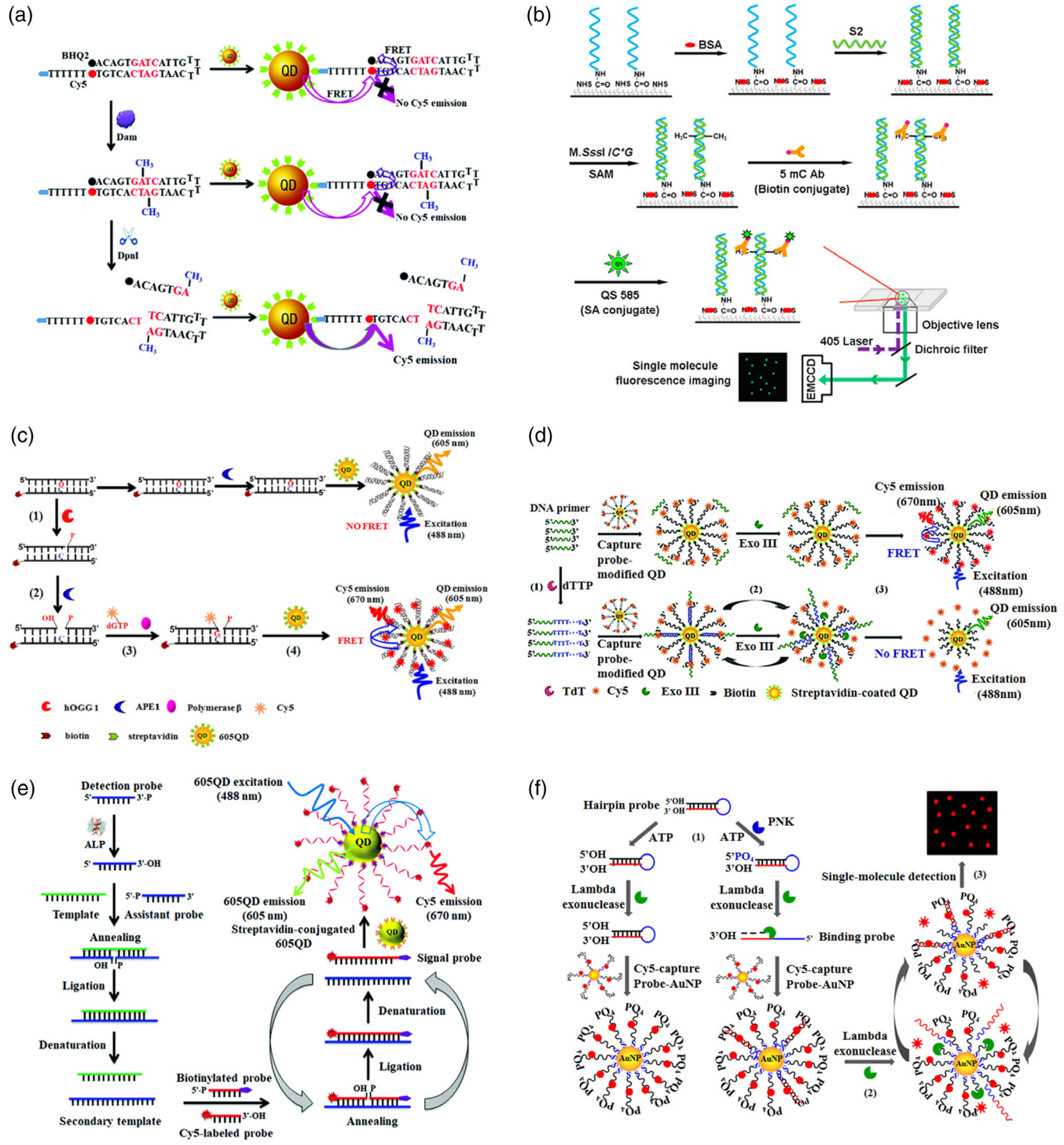

FIGURE 4 (a) QD-based single-molecule fluorescent nanosensor for Dam activity detection. Reprinted with permission from Ma, Liu, et al. (2017). Copyright 2017 The Royal Society of Chemistry. (b) QD-based single-molecule fluorescent nanosensor for M.SssI activity detection. Reprinted with permission from H. Zhang, Zhang, Yao, et al. (2019). Copyright 2019 American Chemical Society. (c) QD-based single-molecule fluorescent nanosensor for hOGG1 activity detection. Reprinted with permission from L.-J. Wang et al. (2016). Copyright 2016 American Chemical Society. (d) QD-based single-molecule fluorescent nanosensor for TdT activity detection. Reprinted with permission from L.-J. Wang, Luo, et al. (2017). Copyright 2017 The Royal Society of Chemistry. (e) QD-base single-molecule fluorescent nanosensor for ALP activity detection. Reprinted with permission from Ma, Liu, and Zhang (2019). Copyright 2019 The Royal Society of Chemistry. (f) AuNP-based single-molecule fluorescent nanosensor for PNK activity detection. Reprinted with permission from L.-J. Wang, Zhang, et al. (2017). Copyright 2017 American Chemical Society

various human diseases including cancers (Jeppesen et al., 2011; Wallace et al., 2012). Zhang et al. constructed a single QD-based fluorescent nanosensor to quantitatively detect human 8-oxoguanine DNA glycosylase (hOGG1) activity (L.-J. Wang et al., 2016). As shown in Figure 4(c), In the presence of target hOGG1, the 8-hydroxyguanine is removed from the biotin-labeled DNA substrate, generating an AP site. The apurinic/apyrimidinic endonuclease (APE1) can excise the AP site to generate a single nucleotide gap. DNA polymerase β enables the incorporation of Cy5-labeled dGTPs in the gapped sites to obtain the Cy5-labeled DNA substrates which can assemble onto the streptavidin-coated QD surface to produce the QD–DNA substrate–Cy5 complex for the generation of efficient FRET between Cy5 and QD. The FRET signals can be quantified by single molecule detection. In the absence of hOGG1, neither the removal of 8-hydroxyguanine from the DNA substrate nor the incorporation of the Cy5-labeled dGTPs into the DNA substrate can occur, and thus no FRET occurs due to the absence of Cy5 acceptor. This nanosensor enables sensitive detection of hOGG1 with a detection limit of 0.0018 U/mL, and it can accurately measure cellular hOGG1 activity in cancer cell extracts. In addition, Li et al. developed a QD-based single-molecule fluorescent nanosensor in combination with APE1-assisted cyclic cleavage-mediated signal amplification to sensitively measure DNA glycosylase hAAG activity with a detection limit of as low as 4.42×10^{-9} U/mL (C.-C. Li et al., 2019).

TdT can catalyze the random incorporation of nucleotides into the 3′-OH termini of DNA strand without the involvement of any DNA templates (Komori et al., 1993) and it is a potential biomarker of leukemic disease (Sur et al., 2007). Wang et al. constructed a QD-based single-molecule fluorescent nanosensor to detect TdT activity (L.-J. Wang, Luo, et al., 2017). As shown in Figure 4(d), he Cy5-labeled A-rich capture probe is assembled on the 605QD surface, and efficient FRET from 605QD to Cy5 can occur in the absence of target TdT. In contrast, TdT can catalyze the incorporation of dTTPs into 3′-end of DNA primer to produce long poly-T sequence which can subsequently hybridize with the A-rich capture probe to obtain duplex DNA. Upon the addition of exonuclease III (Exo III), the capture probes in the duplexes are cyclically digested, leading to the dissociation of Cy5 from QD surface and the decrease of FRET efficiency. Due to the integration of Exo III-assisted signal amplification with single-molecule detection, this nanosensor can sensitively measure TdT activity with a detection limit of 0.001 U/mL, and it can be further employed to quantify TdT activity in human leukemic T cells.

Alkaline phosphatase (ALP) can catalyze the removal of phosphate group from a variety of phosphorylated biomolecules under alkaline conditions (U. Sharma et al., 2014). The aberrant ALP level have been closely associated with different human diseases including bone and hepatobiliary diseases (Rao et al., 2017). Ma et al. constructed a QD-based single-molecule fluorescent nanosensor to detect ALP activity (Ma, Liu, & Zhang, 2019). In the presence of target ALP, 3′-phosphate termini (3′-P) of detection probe is dephosphorylated to obtain a 3′-OH terminus (Figure 4(e)). With the assistance of thermal stable ligase, the 5′-phosphorylated assistant probe is ligated with 3′-hydroxylated detection probe to produce a secondary template. After denaturation, the cyclical ligation of biotinylated probe with Cy5-labled probe induces the production of abundant biotin/Cy5-dual labeled probes. The assembling of biotin/Cy5-dual labeled probes on the streptavidin-conjugated 605QD surface can cause efficient FRET between 605QD and Cy5. Taking advantages of single-molecule detection and ligation amplification reaction, this nanosensor can detect as low as 5.63×10^{-7} U/mL ALP, and it can be employed to analyze the ALP kinetic parameters and measure endogenous ALP activity in different human cell lines.

The 5′-polynucleotide kinase (PNK) can transfer γ-phosphate group to the 5′-OH group of oligonucleotides from adenosine triphosphate (ATP) (Novogrodsky et al., 1966). Dysregulation of PNK activity may disturb the DNA repair system and induces different human diseases (S. Sharma et al., 2006). Wang et al. constructed a AuNP-based single-molecule fluorescent nanosensor to detect PNK activity (L.-J. Wang, Zhang, et al., 2017). As shown in Figure 4(f), the assembly of Cy5-labeled capture probes on AuNP induces the quenching of Cy5 signal by AuNP. The presence of target PNK induces the formation of 5′-phosphorylated hairpin probes which can be specifically cleaved by lambda exonuclease to unfold the hairpin structure and release the binding probes. The hybridization of binding probes with the capture probes can trigger cyclical lambda exonuclease-mediated cleavage of capture probes, resulting in the dissociation of Cy5 molecules from AuNPs. The released Cy5 molecules can be measured by single-molecule detection. This detection limit of nanosensor can reach 9.77×10^{-8} U/μL, and it can be employed to screen PNK inhibitors and quantify endogenous PNK activity in human embryonic kidney HEK293T cell line.

3.5 | Detection of protein modifying enzymes

Protein kinases enables the transfer of γ-phosphate group to specific amino acids in target proteins (Arthur & Ley, 2013), and the deregulation of protein kinase activities is closely linked to different human diseases including

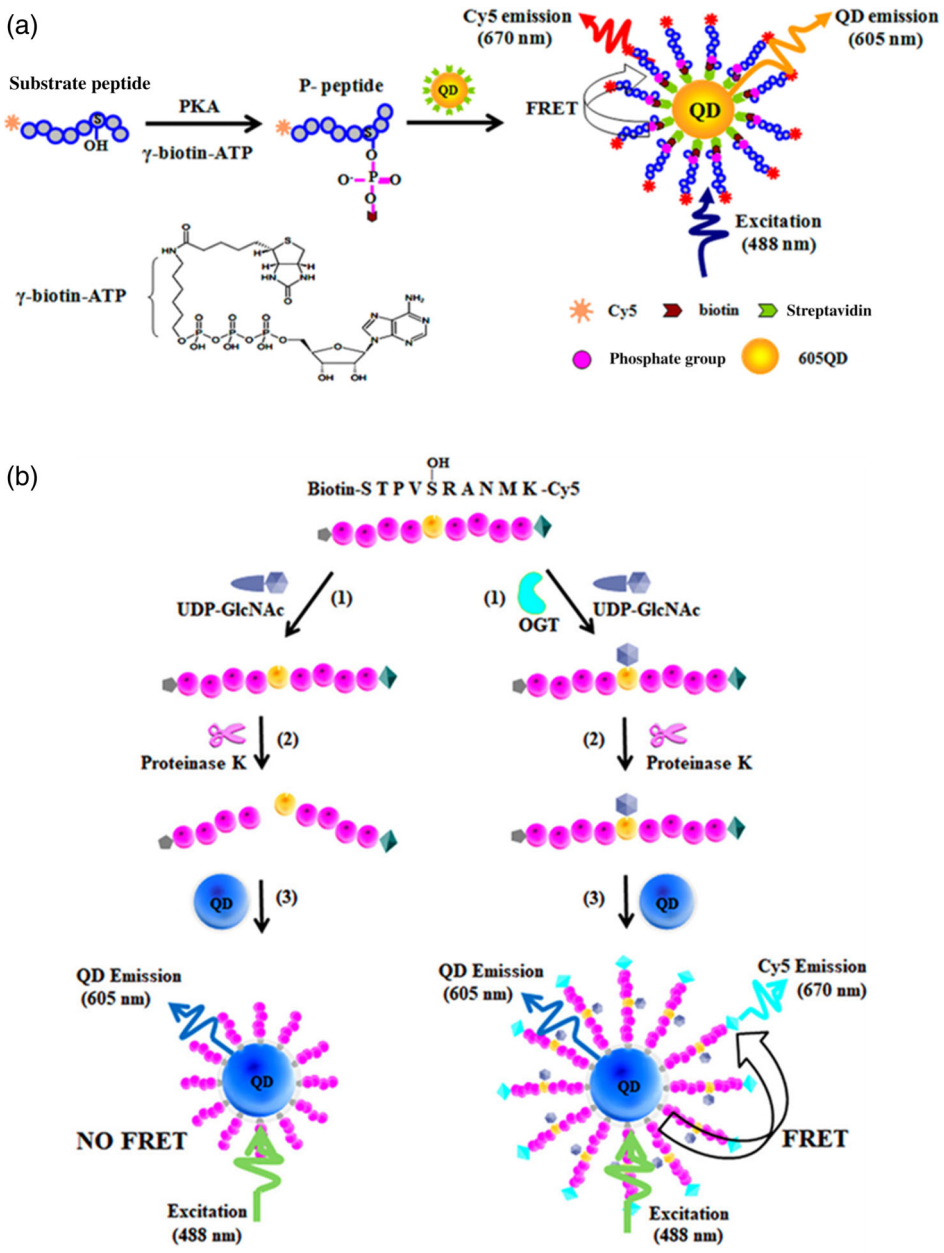

FIGURE 5 (a) QD-based single-molecule fluorescent nanosensor for PKA activity detection. Reprinted with permission from L.-J. Wang, Yang, and Zhang (2015). Copyright 2015 American Chemical Society. (b) QD-based single-molecule fluorescent nanosensor for OGT activity detection. Reprinted with permission from Hu et al. (2017). Copyright 2017 American Chemical Society

cancers (Liu & Winey, 2012; Zaha & Young, 2012). Zhang et al. constructed a single QD-based fluorescent nanosensor to sensitively detect protein kinase (PKA) activity (L.-J. Wang, Zhang, et al., 2015). As shown in Figure 5(a), in the presence of target PKA, the biotinylated γ-phosphate in γ-biotin-ATP is transferred to serine hydroxyl group in peptide substrate, leading to the labeling of peptide substrate with biotin moiety. Subsequently, the biotinylated peptide substrates can assemble the streptavidin-coated QD surface to bring QD and Cy5 into close proximation, inducing efficient FRET between QD and Cy5. In the absence of PKA, neither phosphorylation nor biotinylation of peptide substrates can occur, and thus no FRET is observed. The detection limit of this nanosensor can reach 9.3×10^{-6} U/μL, and it can be further applied for the screening PKA inhibitors and measurement of PKA activity in cancer cells.

The O-linked *N*-acetylglucosamine transferase (OGT) can catalyze the generation of GlcNAc-β-Ser/Thr linkage through transferring a single OGlcNAc moiety to a threonine/serine residue in proteins from UDP-GlcNAc (Moremen et al., 2012), and the deregulation of OGT activity is closely linked to different human diseases such as cancers and

Alzheimer disease (Slawson & Hart, 2011; X. Yang et al., 2008; Yuzwa & Vocadlo, 2014). Zhang et al. constructed a single QD-based fluorescent nanosensor to detect OGT activity (Hu et al., 2017). As shown in Figure 5(b), target OGT enzyme catalyzes the transfer of single sugar GlcNAc to the serine hydroxyl group of the peptide substrate from UDP-GlcNAc, producing a glycosylated peptide substrate. The resultant glycosylated peptide substrates are resistant to proteinase K, and they can assemble on the streptavidin-coated QD surface to form a Cy5–peptide–QD complex, inducing efficient FRET between Cy5 and QD. When OGT is absent, the Cy5/biotin-modified peptide substrates cannot be glycosylated and they are cleaved by proteinase K, leading to the dissociation of Cy5 from QD. As a result, no FRET is observed. This nanosensor is very simple and its detection limit can reach 0.347 pM. Moreover, this nanosensor can be further employed to screen OGT inhibitor and quantify cellular OGT activity in cancer cells.

3.6 | Detection of other enzymes

Glutathione S-transferase (GST) can catalyze the reduced glutathione (GSH) to attack the electrophilic centers, and the aberrant GST level is closely linked to kidney-related diseases (Tew & Townsend, 2012). Xiao et al. developed a single-molecule fluorescent nanosensor to detect GST activity (Han, Chen, et al., 2019). As shown in Figure 6(a), the FCPNPs are modified with glutathione (FCPNPs-GSH) and the AUNPs are capped with polyethylenimine (AUNPs@PEI). In the absence of GST, the FCPNPs-GSH can assemble with the AUNPs@PEI via electrostatic interaction, leading to efficient FRET between FCPNP-GSH and AUNPs@PEI and consequently the quenching of FCPNP signal by AUNP. When GST is present, the FCPNPs-GSH preferentially bind with GST via GST-GSH interaction, inducing the dissociation of FCPNPs-GSH from AUNPs@PEI and the recovery of FCPNP fluorescence signal. This nanosensor exhibits a large linear range of 0.01–6 μg/mL and a detection limit of 1.03 ng/mL, and it can be further applied to accurately detect GST in urine samples.

Acetylcholinesterase (AChE) exists the activities of both aminopeptidase and carboxypeptidase, and it can catalyze the hydrolysis of neurotransmitter acetylcholine (ACh) to produce choline and acetate (Miao et al., 2010). The AChE activity is closely associated with Alzheimer's disease (AD) and the inhibition of AChE activity has become an effective way for symptomatic AD treatment (Dvir et al., 2010). Xiao et al. developed a FCPNPs and manganese dioxide (MnO_2)-based single-molecule fluorescent nanosensor for label-free quantitative detection of AChE activity (Y. Han, Ye, et al., 2019). As shown in Figure 6(b), when target AChE is absent, the fluorescence of FCPNPs is quenched by MnO_2 nanosheets as a result of FRET from FCPNPs to MnO_2 nanosheets. When target AChE is present, the ATCh is hydrolyzed to thiocholine (TCh). TCh reduces MnO_2 to Mn^{2+}, leading to the abolishment of FRET between MnO_2 nanosheets and FCPNPs and consequently the recovery of FCPNP fluorescence. The fluorescent FCPNP can be quantified by single-particle enumeration. This nanosensor enables the accurate measurement of AChE with a detection limit of 1.02 μU/mL. Moreover, it can be further used to scree AChE inhibitors and detect the spiked AChE in human serum samples.

3.7 | Detection of intact viruses and live cells

The virus such as the coronavirus disease 2019 (COVID-19) infection has become a serious issue in the world (X. Cao, 2020). The accurate detection of viruses is essential to clinical diagnosis and treatment of virus-caused diseases (X. Zhang, Zhang, Luo, et al., 2019). Zhang et al. constructed a single-molecule fluorescent nanosensor to simultaneously detect multiple viruses using fluorescent magnetic multifunctional nanospheres (Z. Wu et al., 2019). As shown in Figure 7(a), the fluorescent magnetic multifunctional nanospheres are obtained by the assembly of different-colored QDs and magnetic nanoparticles onto the copolymer nanospheres, and they can serve as both the signal labels and the capture carriers. The red, yellow, and green fluorescent magnetic nanospheres (termed as GMNs, YMNs, and RMNs) are employed to labeled the antibodies against H7N9, H1N1, and H9N2 avian influenza viruses (AIV), respectively. The nanosphere-to-virus ratio is controlled to ensure at least one virus can be conjugated to each nanosphere. Target virus can be captured by the corresponding nanosphere, and the released virus–nanosphere complex can further bind antibody-modified micropore through sandwich immunoreaction, followed by quantification with single-molecule detection. This nanosensor enables simultaneous detection of three viruses (i.e., H7N9, H1N1, and H9N2) at single-particle level with distinct advantages of high specificity and strong anti-interference capability.

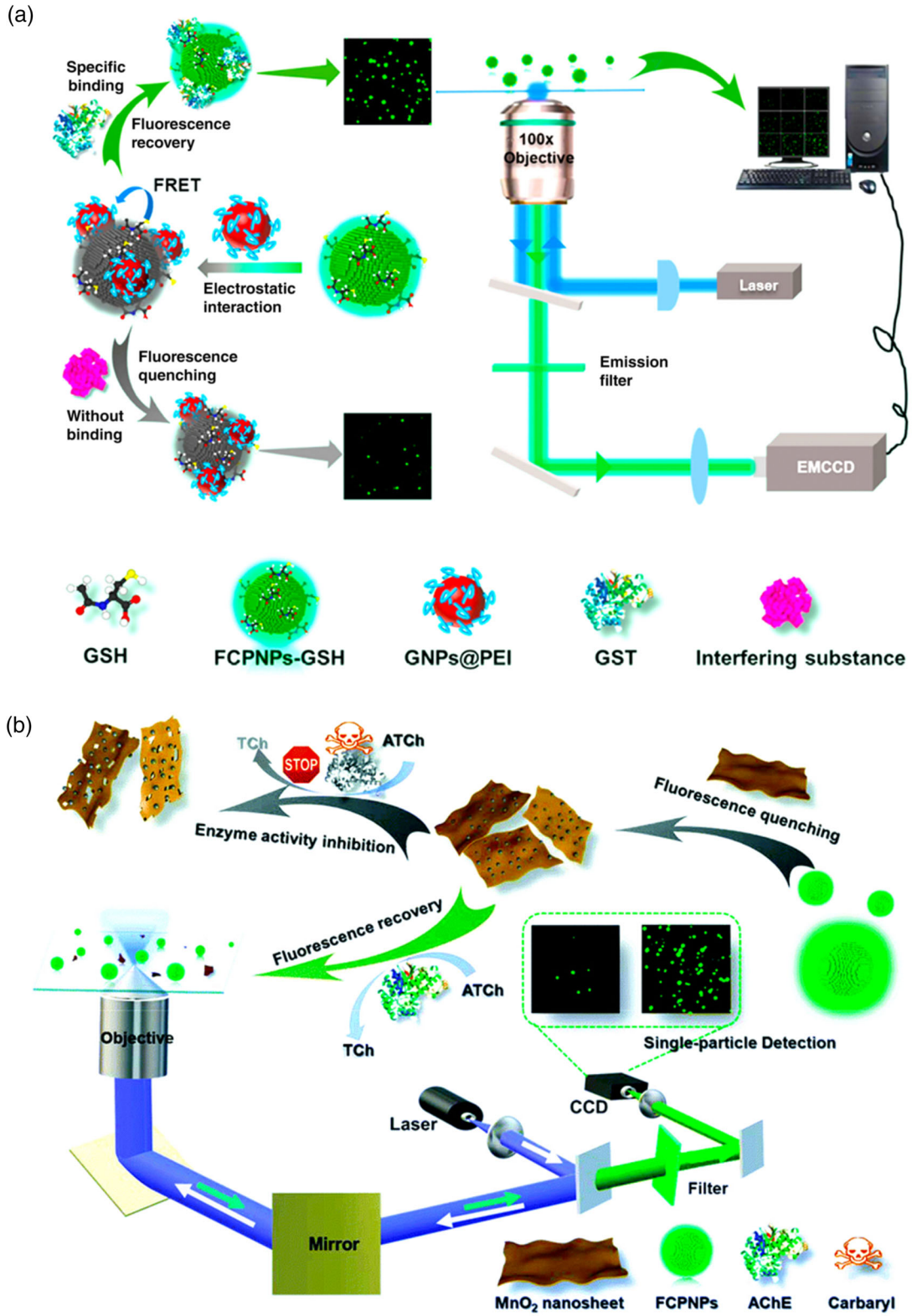

FIGURE 6 (a) FCPNP-based and AUNP-based single-molecule fluorescent nanosensor for GST activity detection. Reprinted with permission from Y. Han, Chen, et al. (2019). Copyright 2019 American Chemical Society. (b) FCPNP and MnO_2 nanosheet-based single-molecule fluorescent nanosensor for AChE activity detection. Reprinted with permission from Y. Han, Ye, et al. (2019). Copyright 2019 The Royal Society of Chemistry

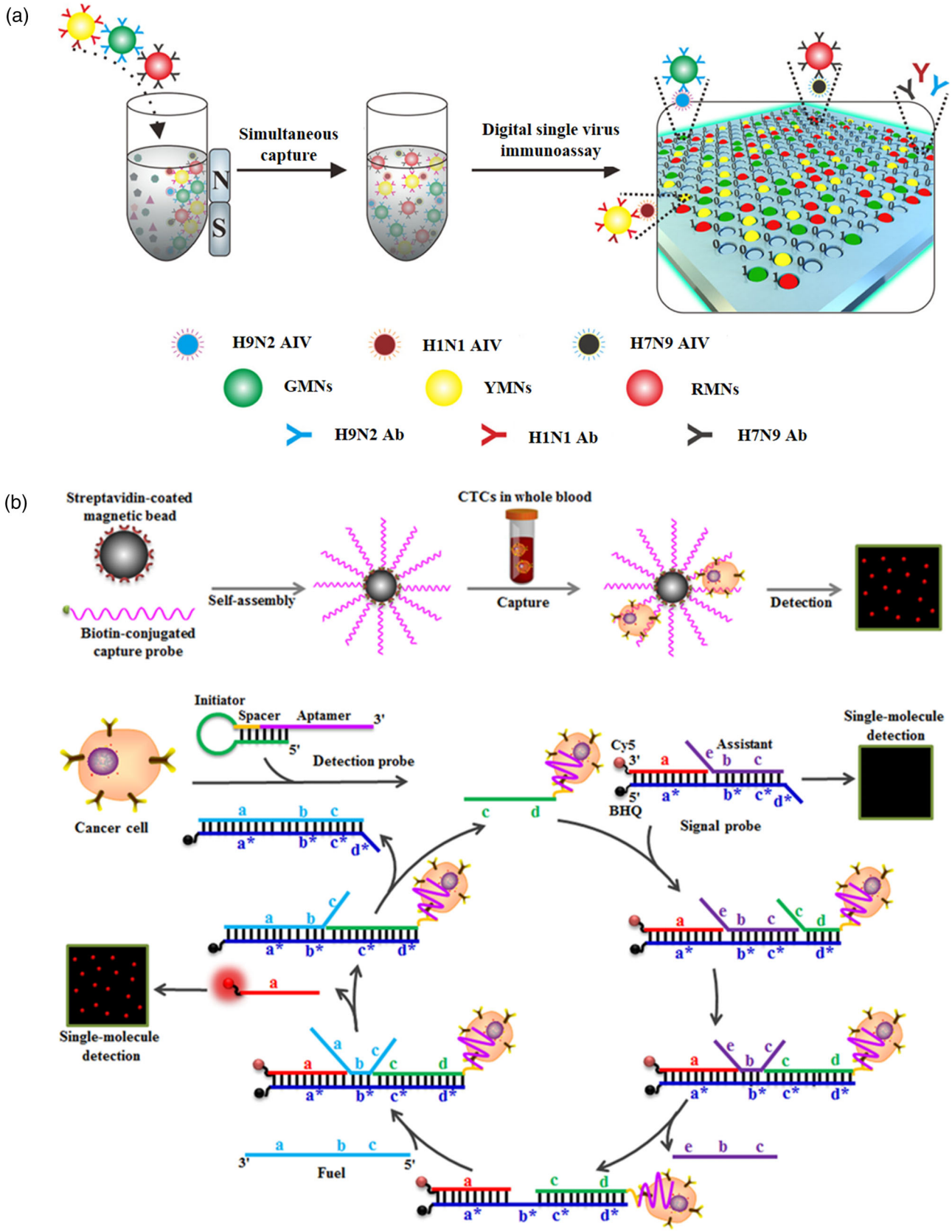

FIGURE 7 (a) Fluorescent magnetic nanospheres-based single-molecule fluorescent nanosensor for multiple viruses assay. Reprinted with permission from Z. Wu et al. (2019). Copyright 2019 American Chemical Society. (b) Magnetic bead-based single-molecule fluorescent nanosensor for cancer cells detection. Reprinted with permission from Ma, Wei, and Zhang (2019). Copyright 2019 American Chemical Society

TABLE 1 Summary of the reported single-molecule fluorescent nanosensors

Nanomaterials	Function	Target	LOD	References
QD	Fluorescent emitter	DNA	1.0 fM	Z. Wang et al. (2012)
QD	Fluorescent emitter	HIV DNA	1 aM for HIV-1 and 2.5 aM for HIV-2	J. Zhou et al. (2013)
FNP	Fluorescent emitter	DNA	2 pM	Pei, Yin, et al. (2018)
FNP	Fluorescent emitter	DNA	—	Pei et al. (2018)
QD	Fluorescent emitter	let-7a microRNA	5 fM	W. Li et al. (2016)
QD	FRET donor	let-7a microRNA	0.1 aM	Zhang and Zhang (2011)
QD	FRET donor	miR-21 microRNA	1 fM	Ma, Zhang, and Zhang (2019)
AuNP	Fluorescent quencher	miR-21 microRNA	1 fM	B. Li et al. (2020)
QD	Fluorescent emitter	miR-155 and miR-21 microRNAs	100 fM	Ma, Zhang, and Zhang (2020)
QD	FRET donor	5-methylcytosine DNA methylation	1.0 aM	Z.-Y. Wang et al. (2018)
QD	FRET donor	Dam MTase	0.002 U/ml	Ma, Liu, et al. (2017)
QD	FRET donor	M.SssI MTase	0.00021 U/ml	Hu et al. (2020)
QD	Fluorescent emitter	M.SssI MTase	0.0005 U/ml	H. Zhang, Zhang, Yao, et al. (2019)
QD	Fluorescent emitter	Thrombin	0.8 pM	J. Liu et al. (2013)
UCNP and AuNP	Fluorescent emitter and fluorescent quencher	PSA	1.0 pM	X. Li et al. (2018)
QD	FRET donor	hOGG1	0.0018 U/ml	L.-J. Wang et al. (2016)
QD	FRET donor	hAAG	4.42×10^{-9} U/ml	C.-C. Li et al. (2019)
QD	FRET donor	TdT	0.001 U/ml	L.-J. Wang, Luo, et al. (2017)
QD	FRET donor	ALP	5.63×10^{-7} U/ml	Ma, Liu, and Zhang (2019)
AuNP	Fluorescent quencher	PNK	9.77×10^{-5} U/ml	L.-J. Wang, Zhang, et al. (2017)
QD	FRET donor	Telomerase	185 cells	J. Zhou, Yang, and Zhang (2015)
QD	FRET donor	PKA	0.0093 U/ml	L.-J. Wang, Zhang, et al. (2015)
QD	FRET donor	OGT	0.347 pM	Hu et al. (2017)
FCPNP and AuNP	Fluorescent emitter and fluorescent quencher	GST	1.03 ng/ml	Y. Han, Chen, et al., 2019
FCPNPs and MnO_2 nanosheet	Fluorescent emitter and fluorescent quencher	AChE	1.02×10^{-6} U/ml	Y. Han, Ye, et al., 2019
MB	Separator and carrier	A549 cell	3 single cell	Ma, Wei, and Zhang (2019)
MB and FNP	Separator and fluorescent emitter	Avian influenza virus	0.01 pg/ml	Z. Wu et al. (2019)

Cancer has posed great threat to human being with 10 million deaths each year (Bray et al., 2018), and the detection of circulating tumor cells (CTC) may facilitate early clinical diagnosis (Haber & Velculescu, 2014). Zhang et al. constructed a MB-based single-molecule fluorescent nanosensor to isolate and detect CTC (Ma, Wei, & Zhang, 2019). As

shown in Figure 7(b), a 5′ biotinylated aptamer is used as the capture probe and is assembled on streptavidin-coated MB to obtain the capture platform with 72% capture efficiency. The captured cells can trigger the activation of entropy-driven DNA nanomachine, leading to the dissociation of Cy5-labeled oligonucleotides from the quenched signal probe and the recovery of Cy5 signal. The Cy5 signal can be measured by single-molecule detection. This nanosensor enables simple and sensitive detection of rare cancers without the requirement of any enzymes and antibodies. This nanosensor can sensitively measure human lung cancer A549 cells with a detection limit of 1 cell, and it can be employed to measure circulating A549 cells in human whole blood with a detection limit of 3 cells.

4 | CONCLUSION

Single-molecule detection is characterized by low sample consumption, good selectivity, and ultrahigh sensitivity, enabling straightforward and visual monitoring of various target molecules (Y. Han, Chen, et al., 2019; B. Li et al., 2020; Ma, Wei, & Zhang, 2019; Pei et al., 2018; Z. Wu et al., 2019). To further improve the performance of the single-molecule detection, a variety of nanomaterials such as QD, AuNP, FCPNP, UCNP, MnO_2 nanosheet, and MB have been introduced for the construction of novel single-molecule fluorescent nanosensors. In these nanosensors, the nanomaterials are rationally modified with different probes (e.g., DNA, peptide, antibody, and small molecule), and they may function as fluorescent labels (e.g., QD, FCPNP, UCNP), fluorescent quencher (e.g., AuNP, nanosheet), and magnetic separator (e.g., MB) (Table 1). These single-molecule fluorescent nanosensors have been successfully employed to detect various disease-related DNAs, microRNAs, proteins, enzymes, live cells, and viruses (Y. Han, Chen, et al., 2019; Y. Han, Ye, et al., 2019; B. Li et al., 2020; W. Li et al., 2016; X. Li et al., 2018; Ma, Wei, & Zhang, 2019; Pei et al., 2018; Z. Wu et al., 2019; Zhou et al., 2013), with wide applications in biomedical research, drug discovery, and clinical diagnosis.

Despite the exciting progress in single-molecule fluorescent nanosensors, there are still some challenges: (a) With the rapid development of nanotechnology and material science, large amounts of nanomaterials have been designed and synthetized, but only parts of them have been exploited in single-molecule fluorescent nanosensors. More efforts should be put into extending the applications of other functional nanomaterials, for example, graphene oxides and carbon nanotubes as efficient fluorescent quenchers (Baptista et al., 2015), graphene quantum dots, silver nanoclusters, and copper nanocluster as the fluorescent labels (Qing et al., 2019; Yeh et al., 2010), and metal–organic frameworks as the separators and probe carriers (Gole et al., 2011), in the construction of single-molecule fluorescent nanosensors. (b) FRET is the most frequently used technology in single-molecule fluorescent nanosensor, and it enables homogeneous detection of target biomolecules (Ma, Liu, & Zhang, 2019; Ma, Zhang, & Zhang, 2019; Z.-Y. Wang et al., 2018). However, most FRET systems require the participation of organic dyes (e.g., Cy5, and Cy3) besides nanomaterials, and they cannot exclude the limitations of organic dyes. The development of FRET-based single-molecule fluorescent nanosensors with the involvement of only functional nanomaterials is highly required. (c) Because different nanomaterials possess diverse properties, the use of different kinds of nanomaterials may efficiently offset the insufficiency of individual nanomaterial and obtain better assay performance than that using only one type of nanomaterial (Lei & Ju, 2012). However, most of single-molecule fluorescent nanosensors only exploit one kind of nanomaterials for detection, and the development of novel biosensing systems which integrate different types of nanomaterials is highly required. (d) The in vivo imaging of intracellular biomolecules may facilitate the real-time monitoring of the dynamics and metabolism of interested biomolecules during cellular pathological and physiological processes (Johnsson & Johnsson, 2007; Smith & Gambhir, 2017), however, only few single-molecule fluorescent nanosensors have been constructed for in vivo imaging of microRNAs, and two typical examples include the QD-based single-molecule fluorescent nanosensors using toehold-mediated strand displacement cascade (Ma, Zhang, & Zhang, 2019) and the AuNP-based single-molecule fluorescent nanosensors using toehold-mediated strand displacement cascade (B. Li et al., 2020). Prior to the in vivo applications of single-molecule fluorescent nanosensors, the investigation of long-term biotoxicity, biocompatibility, stability, metabolic mechanisms, and dynamic behavior of various functional nanomaterials is necessary. With the input of these efforts, single-molecule fluorescent nanosensors will find promising applications in the fields of biochemistry, biology, chemistry, biophysics, drug discovery, and clinical diagnosis in the future.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Meng Liu: Writing-original draft. **Jian-Ge Qiu:** Writing-original draft. **Fei Ma:** Writing-review & editing. **Chun-yang Zhang:** Conceptualization; writing-original draft; writing-review & editing.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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