Chapter 7
Fluorescence Sandwich Assays for Nucleic Acid Detection

Xinwen Liu and Quan Yuan

Abstract Fluorescence sandwich assays have wide application in the detection of nucleic acids due to its well-developed synthesis process, simple detection procedures, and high sensitivity. Specifically, two oligonucleotide probes, named capture probe and signal probe respectively, are introduced and hybridize with different regions of a single-stranded target gene, forming a "capture probe-target-signal probe" sandwiched format. Distinctive fluorescent emission is therefore generated with the formation of sandwich-format and can be directly detected by conventional instruments without further procedures. In this chapter, we conclude the principle and recent developments of this assay based on the classification of fluorophore materials, including fluorescent organic dyes and fluorescent nanomaterials. For each section, the principle of design strategy is firstly introduced, which contains fluorescence resonance energy transfer (FRET) and DNA hybridization-induced fluorescence enhancement. Furthermore, we discuss the limitations and challenges in the development of fluorescent sandwich assays regarding sensitivity and multiple detection capacity, thus providing an overview of the developing situation and offering insight to further developments of nucleic acid assay.

Keywords Fluorescence sandwich assay · Nucleic acid · Organic dye
Quantum dot · Dye-doped nanoparticle

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7.1 Introduction

The obvious advantages of fluorescence-based assay, which include high sensitivity, rapid detection ability, and multiple detection capacity, make it a promising platform for the analysis of nucleic acid. Traditional fluorescence detection techniques are fluorescent in situ hybridization and *Taqman* real-time PCR detection [1–3]. However, laborious labeling, elution procedures, or extra amplification steps are required in such detection process, and the selectivity of analyte is also limited. Therefore, it is important to develop high-sensitivity, simple, and low-cost nucleic acid detection methods. Fluorescence sandwich assay has attracted increasing attention due to its simple design principle, well-developed synthesis methods, and confined detection procedures. Generally, two oligonucleotide probes, named capture probe and signal probe (with fluorophore labeling), are introduced for detection. These two probes hybridize with different regions of a target oligonucleotide sequence, forming a capture-probe-target-signal-probe-sandwiched format. Distinctive fluorescent emission is therefore generated and can be directly detected by conventional instruments without further procedures. Analogous to other fluorescence detection methods, the development of this assay is accompanied with the invention of fluorophores with better properties, the application of different optical effects, and the innovation of detection apparatus, which will be specifically discussed as following.

7.2 Organic Dyes as Fluorophores for Sandwich Assays

Organic dyes have significant application in fluorescence detection due to their availability from commercial sources, small size, and compatibility with various covalent coupling strategies [4, 5]. Moreover, for nucleic acid analysis, organic dyes can be attached at the terminus or any internal position of nucleic acid through suitable linker arms [4]. The well-developed conjugation methods greatly benefit the design of fluorescence sandwich assay for nucleic acids' detection.

In this assay, the strategies that employ organic dye as fluorophore can be divided into two main categories, one is with labeled dyes and the other is the label-free method [4]. The former strategy usually exploits fluorescence resonance energy transfer (FRET) or excimer formation optical effect; while in the latter strategy, intercalation method is frequently used.

7.2.1 Sandwich Assays Based on Fluorescence Resonance Energy Transfer (FRET)

FRET is a non-radiative process contains energy transfer between an excited state donor and a ground state acceptor [6]. As this process stems from dipole–dipole interactions, it is extremely dependent on the separation distance between donor and acceptor (R). Moreover, it also requires a suitable orientation relationship of donor and acceptor, and an appropriate spectral overlap between donor emission and acceptor absorption [5]. The transfer efficiency of FRET is proportional to the inverse sixth power of the molecule distance (R^{-6}), the twice power of orientation factor (κ^2), and also linearly proportional with the spectral overlap integral (J) [6]. This can be expressed in the following equation, where R_0 is the Förster distance, n is the medium refraction index, $f_D(\lambda)$ is the donor emission spectrum, and $\varepsilon_A(\lambda)$ is the acceptor absorption spectrum.

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \tag{7.1.1}$$

$$R_0^6 = \frac{9000(\ln 10)\kappa^2 \phi J}{128\pi^5 n^4 N_{AV}} \tag{7.1.2}$$

$$J = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \tag{7.1.3}$$

In particular, for nucleic acid sandwich assay, two oligonucleotide probes, which are complementary to adjacent regions of nucleic acid target, are modified with a donor and an acceptor at the 3′ or 5′ terminus, respectively. Without the target, because of the low concentration of probes, the distance between randomly diffused acceptors and donors is not close enough for FRET. Therefore, the donor emission is unaffected by acceptors. Upon the target binding, the two probes hybridize with adjacent positions of the target, which brings them in close proximity for FRET. When exciting the donor groups, energy is transferred from donors to acceptors, thus decreasing the signal from donors. This model is also referred to as binary or adjacent probes in DNA hybridization, first reported by Heller and Morrison [7]. The donor should be fluorophore to provide excitation energy in FRET, but the acceptor can be either quencher or fluorophore. Quenchers are not fluorescent and therefore cause the fluorescence of donors simply to decrease [3]. In other words, it is a "signal-off" assay, whose limitation is that the signal change can be over 100% (Fig. 7.1, left) [8]. On the other hand, when the acceptor is fluorescent, it absorbs the energy from donor, which results in the decrease of donor fluorescence and the increase of acceptor fluorescence, making it a "signal-on" assay (Fig. 7.1, right). As two measureable parameters are created in the latter assay, it has wider application compared with the signal-off assay.

Fig. 7.1 Sandwich assay for
nucleic acids based on FRET
(left, middle) and an
intercalation dye (right).
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Quench   FRET   Intercalation
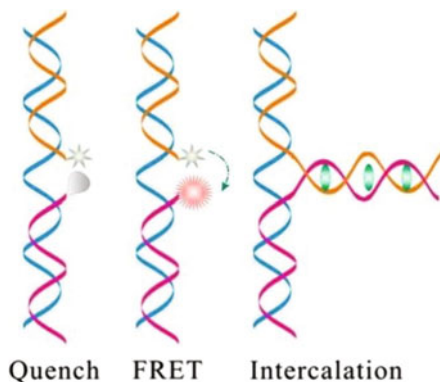
Organic dyes are the first generation of donor–acceptor pairs in FRET appli-
cations, tested with novel fluorescent materials [4, 5]. For nucleic acid sandwich
assays, typical donor fluorophores are cyanine dye (Cy3) and 6-carboxyfluorescein
(6-FAM); the acceptor fluorophores include the cyanine dye (Cy5), 6-carboxy-N,N,
N′,N′-tetramethylrhodamine (TAMRA), and Bodipy493/503 [9]. To maximize the
transfer efficiency, the distance between acceptor and donor is recommended with
1–5 bases. Too close distance might lead to direct interaction between two fluor-
ophores, decreasing the signal intensity [10]. Suitable spectrum overlap between
acceptor and donor fluorophores is also required. These are important factors that
need to be taken into account of in the design of each probe. As for application, the
organic dye-based sandwich assay has been used to detect quantity,
single-nucleotide polymorphism, fragment secondary structure, gene translocation
process, and imaging observation of nucleic acid target [10–14]. For instance, Tsuji
et al. employed this assay for the observation of messenger RNA imaging in living
cells [10]. Bodipy493/503 and Cy5 were used as donor and acceptor fluorophores,
respectively. When these two probes hybridized with target c-fos mRNA, sandwich
format formed and their close proximity resulted in efficient FRET, leading to the
decrease of donor fluorescence (503 nm) and the increase of acceptor fluorescence
(664 nm). As the efficient acceptor fluorescence signal only occurred in the pres-
ence of target, it was used for target localization under the observation of fluores-
cent microscopy. After injecting streptavidin-modified donor and acceptor probes to
living Cos7 cells, successful imaging and localization of c-fos mRNA in single cell
were achieved.

Despite the widespread application of this sandwich assay, the main problem is its
rather high background noise, caused by the direct excitation of non-hybridized
acceptor probes. Although it is a possible solution to reduce the spectral overlap
between the donor and acceptor, the intensity of the FRET signal also correspond-
ingly decreases, as shown in Eqs. 7.1.1 and 7.1.2. Three-dye binary probes have
been invented to improve the signal-to-background (S/B) ratio of this assay, which is
constructed by inserting two fluorophores into the donor probe [6, 15, 16]. The
inserted two fluorophores can be either identical [16] or not [6]. Still employing the

Bodipy493/503-Cy5 pairs, Watanabe et al. labeled two Bodipy493/503 fluorophores on the donor probe. Compared with the ordinary probes, this three-dye system produced a considerable increase in acceptor emission because of the enhanced transfer energy. Moreover, the background noise of this system was also weaker than that of the single-labeled one, possibly due to increased self-quenching of the acceptor fluorophore. Therefore, the S/B ratio was increased [16]. Turro et al. created a FAM–TAMRA–Cy5 FRET system, in which a FAM fluorophore and a TAMRA fluorophore were labeled on the same donor probe and separated by four nucleotides. In the absence of target, because of the close proximity of FAM and TAMRA, strongest fluorescence of intermediate TAMRA could be detected. With the target, energy was transferred from the donor FAM through the intermediate fluorophore TAMRA to the acceptor Cy5, leading to the most intense emission from the acceptor Cy5. Compared with the three-dye system employing identical donor fluorophores, the introducion of an intermediate fluorophore (TAMRA) makes it possible to choose the donor and acceptor with even smaller spectral overlap, reducing the direct excitation and also providing efficient energy reaching acceptor [6].

Another approach is to employ molecular beacons (MBs) in the construction of either donor probe [17] or both the donor and acceptor probes [18]. MBs are dual-labeled hairpin-shaped oligonucleotide probes with a reporter fluorophore at one end and a quencher at the other end. Without the target, the reporter and quencher are brought in close proximity by the stem formation, resulting in the FRET-based quenching of the reporter and thus limiting the background signals. However, in the presence of the target, the probe hybrids with the target and undergoes a conformational reorganization, which disrupts the FRET and restores the fluorescence of donor [17, 18]. When introducing MBs to the sandwich assay, the background signals, caused by current emission of unhybridized donors and direct excitation of acceptors, can be effectively reduced by the intramolecular quenching effect of MBs [9]. Bao et al. designed a sandwich assay employing two MB probes as donor and acceptor, respectively [18]. The donor MB probe was attached with a BHQ-2 quencher to the 3′ end and a Cy3 fluorophore to the 5′ end. The acceptor MB probe was labeled with a BHQ-3 quencher to the 5′ end and a Cy5 fluorophore to the 3′ end (Fig. 7.2). When both the MB probes hybridize with target mRNA and thus undergo conformational change, FRET happens between the Cy3 fluorophore and Cy5 fluorophore. Employing these dual MB probes, localization of the K-ras mRNA expression in living HDF cells was achieved with rather low background interference.

7.2.2 Nucleic Acid Sandwich Assay Based on DNA Hybridization-Induced Fluorescence Enhancement

An excimer is a dimer that is formed by two molecules of the same species. One molecule is in the excited state, and the other is in the ground state. The simplest
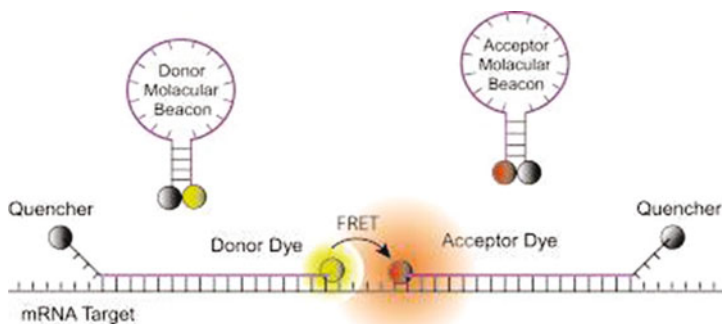
Fig. 7.2 Scheme of dual molecular beacon (MBs)-based nucleic acid sandwich assay. Two MBs function as donor probe and acceptor probe, respectively. In the presence of targets, both the probes undergo conformational reorganization and hybridize with adjacent parts of the target, allowing for FRET between donor dye and acceptor dye. (Reprinted with permission from Ref. [18]. Copyright 2004 Oxford University Press)

formation of an excimer can be shown in equation below ($^1M^*$ is a singlet excited molecule, ^1M is an unexcited molecule, and $^1D^*$ is the excited dimer) [19]. The excited monomer will relax to the steady singlet S^1 state through internal conversion at first and then constitute a lower energy excimer when meeting with a ground state molecule. In the later dissociation process, the lower energy excimer will release a photon at a longer wavelength than the excited monomer does. Because of the characteristic longer wavelength region and broad band of excimer emission, it can be easily distinguished from the monomer fluorescence, allowing for the application of excimer-based probes in real-time hybridization assays [19, 20].

$$^1M^* + {}^1M \leftrightharpoons {}^1D^* \tag{7.2.1}$$

$$^1D^* \rightarrow {}^1M + {}^1M + h\nu_D \tag{7.2.2}$$
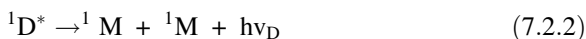
Specifically, when applying excimer formation principle to sandwich assay, the main construction of the sandwiched structure is similar with that of the FRET-based assay. The only difference lies in the choice of labels on probes. Both strands of excimer probes are attached with a monomer molecule which is able to form excimer in close proximity. This assay was also referred to as excimer-forming two-probe hybridization method, firstly reported by Ebata and his coworkers [20]. Pyrene, the well-characterized monomer, was chosen as a fluorophore and was attached on two separated oligonucleotide probes (Fig. 7.3). When these probes hybridized to the target Vibrio mimicus 16S rRNA, a 495-nm broad fluorescence band spectra were easily discriminated from the monomer fluorescence (below 450 nm). With the growing concentration of targets, the intensity of this band increased and that of the pyrene monomer decreased, indicating that the 495-nm band was attributed to the pyrene excimer. A detection limit of 10 nM was achieved in this system [21].
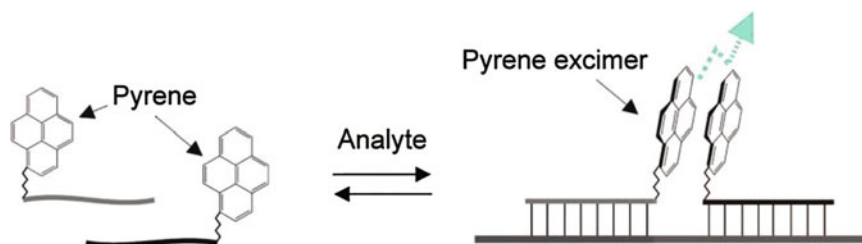
Fig. 7.3 Scheme of dual excimer formation-based nucleic acid sandwich assay. Two target-complementary probes are modified with a pyrene monomer. When hybridizing with target, the close proximity between the two monomer allows the formation of an excimer, generating distinctive fluorescence signal. (Reprinted with permission from Ref. [9]. Copyright 2010 American Chemical Society)

The main advantage of this assay is that excimer's large Stoke shift allows for the use of a wider spectral band-pass filters for the detection channels, which is beneficial to signal intensity enhancement [22]. However, to avoid the quenching effect caused by nearby nucleic acids through photo-induced electron-transfer (PET) process, 20% (v/v) DMF has to be added in the hybridization buffer to provide efficient signal intensity [21], which is not suitable for in vivo applications. Moreover, the excitation wavelength of monomer is at around 340 nm, which will cause limited depth penetration and serious auto fluorescence of cellular environment. Fortunately, another feature of excimers can also be applied to cell studies. Their relatively long lifetime (30–70 ns), in comparison with the short lifetime of the auto fluorescence background in biological sample (8 ns), enables the use of time-resolved fluorescence spectroscopy for signal discrimination [19, 22]. Turro et al. employed this method for mRNA detection in neuronal extracts [22]. Although the cellular extracts showed rather high background noise after 340 nm excitation, the emission decay of this noise was 8 times faster than that of the pyrene excimer probes. The pyrene excimer emission signal was thus gated in the interval of 30–150 ns, giving an S/B ratio of ~ 10.

The aforementioned approaches all involve dye labeling in the construction of oligonucleotide probes, which requires extra chemical synthesis and purify steps and thus improves the cost. To avoid chemical modifications, label-free sandwich methods have been invented with the development of organic dye-specific aptamers. Aptamers are artificial functional oligonucleotides that are able to bind to specific target molecules. They are selected from large random DNA or RNA pools utilizing in vitro selection (SELEX) approach [9]. During the synthesis, only target-binding molecules are isolated as aptamers so that they have great combination ability with targets. They are also known as the strong rival to antibody. Aptamers have been selected toward a broad range of targets, including organic dyes. For instance, malachite green (MG)-binding aptamers were first reported in 1998 [23], which increased the dye fluorescence more than 2000-fold upon binding by stabilizing MG in a planar (more fluorescent) conformation [24]. Kolpashchikov

firstly used this aptamer for sequence detection of nucleic acids [25]. In this work, the RNA aptamer was separated into two strands. Both strands were attached with target-complementary nucleic acid binding arms through UU dinucleotide bridges (Fig. 7.4). Without the DNA target, MG and probes were separated and the fluorescence of MG was comparably weak. In the presence of the target, both strands hybridized with adjacent potions of target and reform the MG aptamer, constructing a non-conical sandwich structure complex. Strong fluorescence increase (~ 20 times higher) was therefore produced due to the enhancement effect of MG aptamers. The detection limit of this system was around of 100 nM.

The development of this assay is accompanied with the invention of aptamers with larger enhancement effect and stronger binding ability. After the production of MG aptamer, Hoechst dye-aptamer [26, 27] has also been synthesized for single-nucleotide resolution, with nearly 143 times fluorescence enhancement compared with direct dye excitation. Recently, an RNA molecule named "Spinach aptamer" with affinity for a fluorescent dye DFHBI was produced, which has great enhancement effect with nearly negligible photobleaching [28]. Exploiting this Spinach system, successful RNA recognition was achieved, which yielded a 270 times higher fluorescence after binding and obtained a LOD at 1.8 nM [29], superior to the existed intercalation-based system.

Conjugated polymers (CPs) are organic macromolecules comprising at least one chain of alternating double bond and single bond. Their delocalized π electron system allows for light absorption and photo-generated charge carrier generation, making them the ideal light-harvesting material [30]. Moreover, the excitation can migrate along the polymer chain through efficient energy transfer. Thus, CPs are promising candidates for FRET donor materials [31].
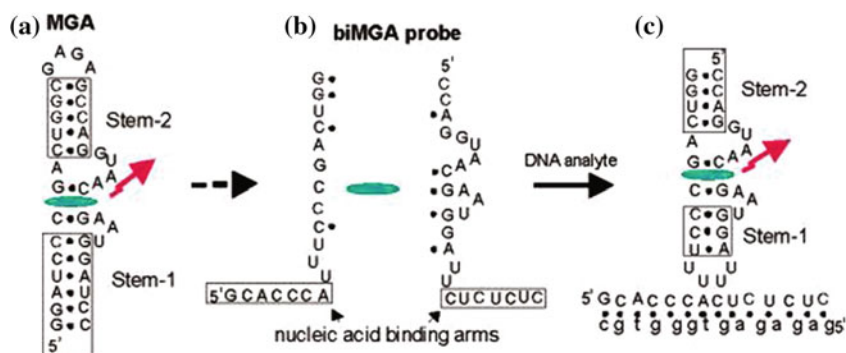

Fig. 7.4 Scheme of intercalation-based nucleic acid sandwich assay (MG aptamer). **a** The structure of MG aptamer. **b** The MG aptamer is separated into two strands, both of which are attached with target-complementary nucleic acid binding arms. **c** In the presence of DNA analyte, the two strands hybridize with target, reforming the MG aptamer. High S/B ratio can be obtained due to the fluorescence amplification effect of MG aptamer. (Reprinted with permission from Ref. [25]. Copyright 2005 American Chemical Society)

In particular, for DNA sandwich assay, a cationic CP functions as the capture probe, and a target-complementary PNA strand labeled with a fluorescent dye acts as the acceptor probe. In the presence of target, the PNA probe forms stable Watson–Crick base pairs with the single-stranded DNA target. The cationic CP then combines with the polyanionic PNA/DNA complex through electrostatic interaction, constructing a probe-target-probe-sandwiched format (Fig. 7.5) [32]. The close proximity of CP and the fluorophore on PNA enables FRET and therefore generating fluorescent signals of the fluorophore. In PNA, the negatively charged phosphate linkages in conventional DNA are replaced with peptomimetic neutral amide linkages, making it a neutral material. Thus, without the DNA target, no electrostatic interaction happens between cationic CP and neutral PNA probe, limiting the background signal.

Bazan et al. firstly reported this system and employed it for quantification detection of target DNA. A 25-times amplification of fluorescein emission compared with direct dye excitation was achieved, with a detection limit of 10 pM [32]. However, to avoid detrimental background noise caused by the hydrophobic interaction of PNA and cationic CP, 10% ethanol solution was added. In order to further improve the sensitivity of this scheme, label-free methods employing positively charged intercalation dye have been invented [33]. Specifically, cationic CP (PFP), target-complementary single-strand DNA (ssDNA) probe and intercalation dye, Genefinder (GF), were mixed in the solution with suitable ratio. When adding
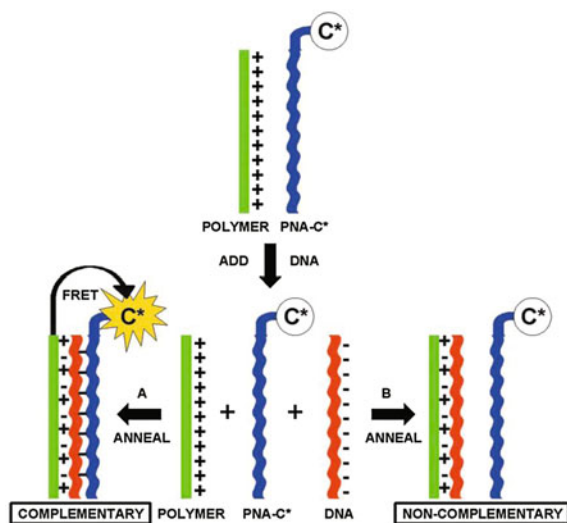

Fig. 7.5 Scheme of conjugated polymer-assisted sandwich assay. Cationic conjugated polymer functions as donor probe, and fluorophore-labeled PNA functions as acceptor probe. With the ssDNA target, target-complementary PNA forms stable Watson–Crick base pairs with the target. Cationic conjugated polymer combines with the polyanionic PNA/DNA complex through electrostatic interaction. The close proximity between polymer and fluorophore on PNA allows for FRET. (Reprinted with permission from Ref. [32]. Copyright 2002 National Academy of Sciences)

the target, PFP and helix DNA formed a sandwich structure and GF dye intercalated into the helix DNA, which brought GF and PFP in close proximity and thus allowed for FRET. Because both PFP and GF are positively charged, electrostatic repulsion between them should be expected without the target, which effectively reduces the background noise and improves its sensitivity. Another label-free method employing pyrene-functionalized polymer and intercalation dye SYBR Green I has also been reported for selectivity promotion [34].

7.3 Nanomaterials as Fluorophores for Sandwich Assays

Although organic dyes have been widely used in fluorescence sandwich assay, they also have certain limitations, such as their self-quenching at high concentrations, susceptibility to photobleaching, and narrow absorption windows with small Stoke shifts, which impede the sensitivity improvement [4, 5].

In comparison with organic dyes, nanomaterial has been developed as the new generation fluorescence material with great optical properties. Specifically, for nucleic acid sandwich assays, quantum dots (QDs) and dye-doped nanoparticles have been applied as fluorophores.

7.3.1 Quantum Dots as Fluorophores

QDs are semiconductor nanocrystals with physical dimensions smaller than the exciton Bohr radius, which have great electronic and optical properties [35]. The advantages of QDs, such as strong fluorescence, high photostability, size-tunable narrow emission, and broad excitation window with large Stoke shifts, make them the most promising fluorescent materials.

In particular, for nucleic acid sandwich assay, the strategies for employing QDs are similar with those of the organic dye-based sandwich assay. They can be mainly divided into two categories, which are using QD as the signal output (Fig. 7.6, left) or as the donor in FRET-based assay (Fig. 7.6, right) [2].

7.3.1.1 Quantum Dots as the Signal Output

In this assay, both the target-complementary capture probe and the report probe are labeled with fluorophores that have distinct emission, exploiting the principle of dual-color fluorescence coincidence detection [36]. These two distinct emissions are used to "encode" the target, since only the sandwiched structure reflects the fluorescence of both the two channels and thus is able to be distinguished from backgrounds. Specifically, for QD-based sandwich assay, the luminescence of QDs functions as the reporting signal, analogous to the barcode in code system. QDs

Fig. 7.6 Strategies for applying quantum dots in DNA sandwich assay. Quantum dots can be used as the signal output (left) or as the donor in FRET-based sandwich assay. (Reprinted with permission from Ref. [2]. Copyright 2014 American Chemical Society)
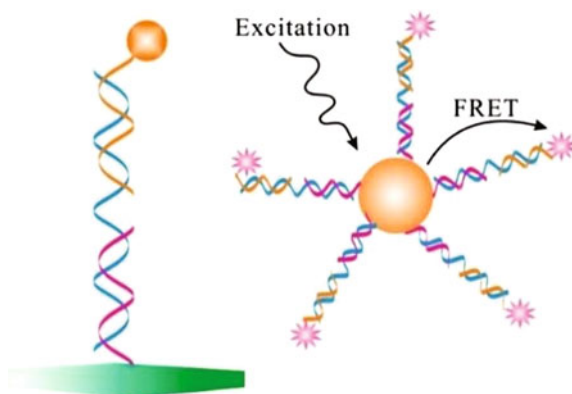
with surface-functionalized oligonucleotide probes can be used as either report probe [37], capture probe [38], or both in this system [39].

Recently, Kim et al. developed the bead-based sandwich hybridization for rapid analysis of the Bacillus spoOA gene [37]. Fluorescent bead-DNA complex functioned as the capture probe and QD 655-DNA, constructed via biotin–streptavidin interaction, was used as the signal probe (Fig. 7.7). Analyzed by flow cytometry, the area of sandwich complex was determined by four parameters, the scan scatter, electronic volume, green fluorescence from bead, and red fluorescence from QDs. This system demonstrated both wider linear range (3.2–1000 nM) and lower detection limit (0.02 nM) than conventional DNA biosensors.

QD-DNA probe can also act as the capture probe, in which QDs are the nanoconcentrators of target that have signal amplification effect [38]. Wang et al. used two ssDNA probes for detection, one modified with biotin and the other labeled with an organic fluorophore, Oregon Green 488 (peak emission at 524 nm). After they form a sandwich complex with target, streptavidin-modified QDs (core–shell CdSe-ZnS, QD 605) were added to capture these biotinylated sandwich structures. Because a single quantum dot was able to combine with several copies of sandwiched target, the signal of organic fluorophore was amplified. Confocal fluorescent spectroscopy was used for target detection, as the coincidence of green channel and red channel only occurred in the presence of targets. This nanobiosensors showed better sensitivity and specificity compared with MB-based assays due to its amplification effect. However, the limitations of organic dyes, such as narrow absorption windows with small Stoke shifts, result in emission spectral overlap (also known as cross talk) between organic dyes and QDs, in addition with the confined choice of excitation wavelength, hindering the next development of multiple targets detection.

Considering the disadvantage brought about by organic fluorophores, Wang et al. firstly employed two QD-DNA probes in the colocalization analysis [39]. These probes have discernible emission wavelengths and hybridized with the target DNA in juxtaposition, forming a sandwiched structure (Fig. 7.8). Analyzed by
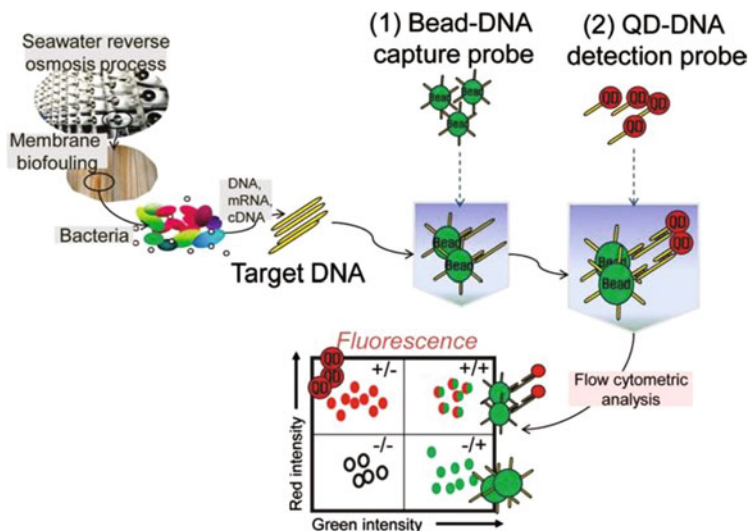
Fig. 7.7 Scheme for employing quantum dots as reporter probe in sandwich assay. Bead-DNA functions as capture probe, and QD-DNA functions as signal probe. Sandwich complex is formed when these two probes hybridize with targets. Only the sandwich complex reflects both green fluorescence from bead and red fluorescence from QDs. (Reprinted with permission from Ref. [37]. Copyright 2010 American Chemical Society)

confocal fluorescent microscopy, only the target showed emission of two channels (presented by pseudocombined color). Exploiting this assay, simultaneous detection of three genes associated with anthrax pathogenicity was achieved at a single molecule level. Moreover, due to the outstanding optical properties of QDs, such as the broad excitation window and size-tunable emission wavelength, the multiple detection capacity of this assay can be further increased by using extra QDs with distinct emissions. Specifically, n different QDs can simultaneously detect $\frac{1}{2}n(n-1)$ targets.

7.3.1.2 Quantum Dots as the Donor in FRET-Based Assays

QDs are promising as FRET donors due to their size-tuned emission and broad absorption window, which avoid spectral cross talk and decrease the direct excitation of acceptors. Also because of their broad absorption width property, it can be inferred that they are not suitable as acceptors. To complete the probe construction, organic dye is frequently used as the acceptor fluorophore in QD-based sandwich assay. Wang et al. firstly employed 605 QD-Cy5 in FRET system [40]. The construction process of sandwich nanoassembly is similar with the group's aforementioned work employing QDs as the nanoconcentrators [38]. The difference lies in whether FRET is involved in the detection. In this 605 QD-Cy5 system, the
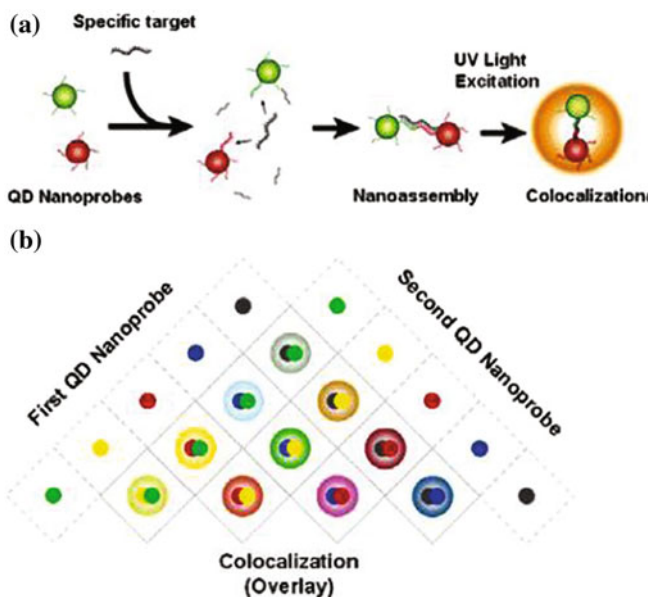
Fig. 7.8 Scheme for employing two QD-DNA probes in a sandwich assay. **a** Scheme model: QDs with distinctive emission wavelengths are introduced to encode the target. **b** The color combination scheme for multiplexed colocalization detection. (Reprinted with permission from Ref. [39]. Copyright 2005 American Chemical Society)

spectral overlap and close proximity between these two fluorophores allow for FRET (Fig. 7.9). Compared with the conventional organic fluorophore system, the direct excitation of Cy5 can be greatly reduced due to the broad excitation window of QDs, which allows the choice of sample excitation wavelength at near the minimum absorption wavelength of acceptors. Besides, the capability of capturing several acceptors by a single quantum dot improves the overall energy-transfer efficiency. This system showed $\sim$100-fold higher sensing signal than that of MBs and achieved a detection limit of 4.8 fM. For further developments, solid-phase immobilization of different QDs with distinct emissions on optical fibers was introduced in this system [41, 42], which enables multiple target detection. Microfluidic chip with separated channels [43] and paper-based analysis [44] were also invented for detection device construction.

Despite the advantages of QDs as FRET donors, the large size of QDs, contributed by the core–shell CdSe-ZnS structure, extra coating, and bioconjugation, impedes the FRET transfer efficiency, which is strongly dependent on the distance R [10]. Considering the "tail-to-tail" configuration in QD-based sandwich assay, only short-length oligonucleotide probes can be used to ensure the transfer efficiency, which may limit further development. To improve the sensing distance range, QD-gold system was invented, in which nanometer gold particles (AuNPs) quench the fluorescence of QDs with high efficiency in larger distance (up to 15–
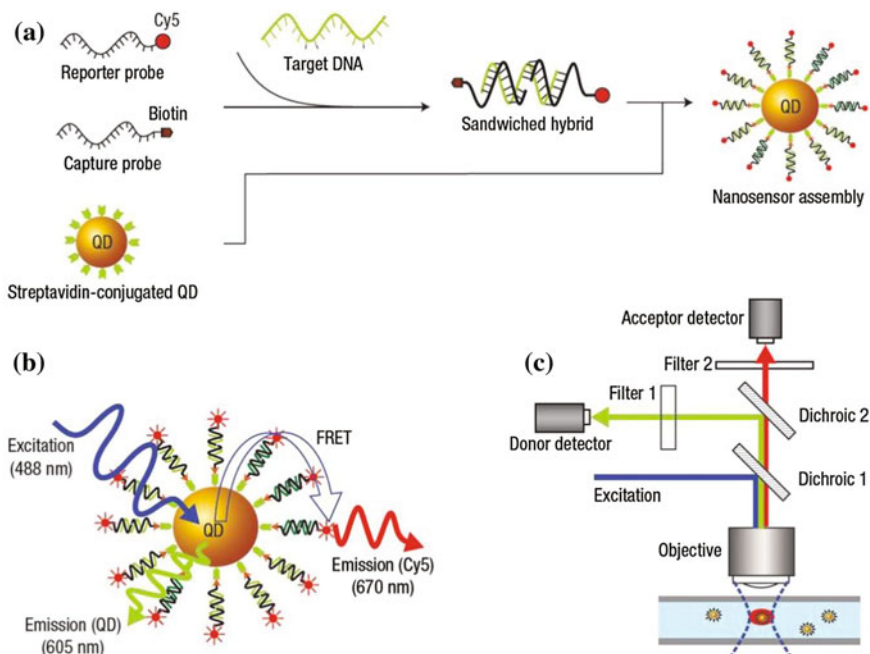
Fig. 7.9 Scheme for quantum dots as donors in FRET-based sandwich assay. **a** Conceptual scheme of sandwich hybrid formation. QDs function as both the signal probe and target concentrator. **b** Scheme for FRET process between QDs and Cy5 fluorophore. **c** Detection apparatus. (Reprinted with permission from Ref. [40]. Copyright 2005 Nature Publishing Group)

20 nm). Although the quenching mechanism has not been completely proved yet, experiments showed that energy transfer from QDs to small size AuNPs (3 nm) had a nanometal surface energy transfer (NSET)-like d^{-4} dependence, which makes larger distance detection available [45]. Lee et al. constructed a head-to-head-sandwiched format in which QD functions as donor and AuNP (5 nm) acts as acceptor. Because of the head-to-head configuration and the possible NSET principle between QDs and AuNPs, oligonucleotide probes with a longer length (30-mer) than the conventional FRET-based detection ($\sim$ 15-mer) could be chosen. Simultaneous detection of two variation types of EML4–ALK fusion genes was achieved by employing green-emitting and red-emitting quantum dots. The detection limit was of 3.45 nM [46].

7.3.2 Dye-Doped Nanoparticles as Fluorophore

In addition with quantum dots, to overcome the aforementioned limitations of organic dye fluorophores, dye-doped nanoparticles have also been greatly

investigated. Nanoparticles function as the "container" of organic fluorophores, which either encapsulate the fluorophore inside them or attach it on their surface [47]. Among different nanoparticles, silica nanoparticle (SiNP) is the ideal carrier and its dye-nanoparticle assembly is frequently used in DNA sandwich assay.

Compared with conventional organic dyes, dye-dope SiNPs have three main advantages. Firstly, as SiNPs encapsulate a large number of dye molecules inside the silica matrix by absorption, the fluorescence signal is greatly amplified, making it possible for ultrasensitive detection. Secondly, the shielding effect of SiNPs increases the stability of organic dyes. Moreover, the flexible silica chemistry provides versatile routes for surface modification, enabling various biomolecule conjugations [48, 49].

Specifically, for conventional DNA sandwich assays, one most important limitation is that a DNA probe can only be labeled with one or a few fluorophores, prohibiting the ultratrace detection of nucleic acids. One solution is to use dye-doped SiNPs with amplification effect. Zhao and his coworkers developed a sandwich assay based on dye-doped SiNPs [48]. In this work, the biotinylated capture probe (DNA 1) was firstly immobilized on an avidin-coated glass substrate. The signal probe (DNA 3) was labeled with a TMR fluorophore-doped SiNP for signal generation. Both these two probes were complementary to the target. When adding the target (DNA 2) and the capture probes in sequence, the capture probe and the signal probe hybridized with different regions of the target, forming a sandwich structure (Fig. 7.10). Because each SiNP was doped with a large number of fluorophore molecules, the generated signal for even one DNA was greatly amplified. Control experiment proved that the SiNP provided an approximately 10^4 times higher signal than that of the simple TMR fluorophore in the same assay. By monitoring the fluorescence image and fluorescence intensity, a detection limit as low as 0.8 fM was achieved.

Although dye-doped SiNPs have obvious advantages, several limitations still exist. Because the organic dye molecules are randomly embedded in the silica matrix, fluorophore aggregation occurs, which lead to the decrease of fluorescent efficiency and variation of the amount of dye molecules encapsulated in each
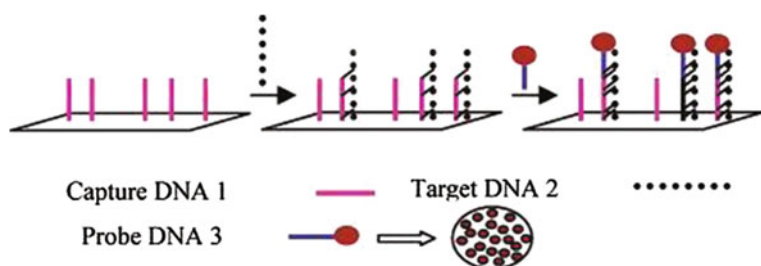

Fig. 7.10 Scheme for dye-doped nanoparticles as fluorophore in a sandwich assay. Biotinylated capture probe is immobilized on the glass substrate; single probe is modified with dye-doped SiNPs. Fluorescence is greatly amplified due to the high fluorophore loading capacity of SiNPs. (Reprinted with permission from Ref. [48]. Copyright 2003 American Chemical Society)

nanoparticle. To prevent such problems, Zhou et al. further developed this assay by creating cyanine dye-doped Au/silica core–shell nanoparticles [50]. The cyanine dyes were firstly conjugated with Au colloidal core through $(dT)_{20}$ oligomers and then embedded in the silica shell, which effectively inhibited the dye blinking problem. The amount of organic fluorophore for each silica shell was also proved to be around of 95 molecules, leading to good reproducibility results. By using Cy3- and Cy5-doped Au/Si NPs in sandwich assay, two-color DNA microarray-based detection was demonstrated and detection limits of 1 pM were obtained.

7.4 Conclusion

In this chapter, we summarized the principle of design approaches, the limitations, and related developments of fluorescence sandwich assay. Great efforts have been made for sensitivity improvement, detection capacity enlargement, and application field expansion. The modification of the oligonucleotide probe, which includes inserting two fluorophores into the capture probe and employing MB as probes, has effectively improved the S/B ratio of FRET-based sandwich assay. The exploiting of different design approaches, such as excimer formation and intercalation, either improves the imaging resolution or simplifies the synthesis procedures. The advent of fluorophores with superior optical properties, including CPs, QDs, and dye-doped nanoparticles, greatly promotes its quantification detection. As low as of 0.8 fM detection limit has been obtained with the application of dye-doped SiNPs. Even more sophisticated detection instruments and techniques offer more opportunities for in vivo studies and multiple target analysis. The utilization of time-resolved fluorescence spectroscopy efficiently decreased the autofluorescence signal of in vivo applications. Fluorescence microscopy achieved the imaging and localization of target in biological samples. As for simultaneous detection of multiple targets, the application of confocal fluorescence microscopy and flow cytometry increased precision level of multiple capacities through multiple parameters determination. For further development of this assay, the synthesis of new fluorescent materials and invention of high-throughput detection techniques are still required. By combining the strategies discussed above, such as exploiting design approaches with appropriate probe modifications, and the use of high property fluorophores with state-of-the-art detection techniques, the fluorescence nucleic acid sandwich assay can be further developed.

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