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Signal Amplification Technologies for the Detection of Nucleic Acids: from Cell-Free Analysis to Live-Cell Imaging

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Abstract Due to their unique properties, such as programmability, ligand-binding capability, and flexibility, nucleic acids can serve as analytes and/or recognition elements for biosensing. To improve the sensitivity of nucleic acid-based biosensing and hence the detection of a few copies of target molecule, different modern amplification methodologies, namely target-and-signal-based amplification strategies, have already been developed. These recent signal amplification technologies, which are capable of amplifying the signal intensity without changing the targets' copy number, have resulted in fast, reliable, and sensitive methods for nucleic acid detection. Working in cell-free settings, researchers have been able to optimize a variety of complex and quantitative methods suitable for deploying in live-cell conditions. In this study, a comprehensive review of the signal amplification technologies for the detection of nucleic acids is provided. We classify the signal amplification methodologies into enzymatic and non-enzymatic strategies with a primary focus on the methods that enable us to shift away from in vitro detecting to in vivo imaging. Finally, the future challenges and limitations of detection for cellular conditions are discussed.

Keywords Nucleic acid nanotechnology · Non-enzymatic detection · Enzymatic detection · Signal amplification · Live-cell imaging · Nucleic acid detection

Abbreviations
ABTS   2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonicacid
AP   Alkaline phosphatase
AuNPs   Gold nanoparticles
bDNA   Branched DNA
CHA   Catalytic hairpin assembly
DNA   Deoxyribonucleic acid
DSN   Duplex-specific nuclease

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dsDNA	Double-stranded DNA
EDC	Entropy-driven catalysis
FISH	Fluorescence in situ hybridization
FRET	Fluorescence resonance energy transfer
GO	Graphene oxide
HCR	Hybridization chain reaction
ISH	In situ hybridization
LOD	Limit of detection
LSPR	Localized surface plasmon resonance
MB	Molecular beacon
MMPs	Magnetic microparticles
NC	Nanocluster
PCR	Polymerase chain reaction
PMP	Polymer microparticles
PO	Horseradish peroxidase
QDs	Quantum dots
SERS	Surface-enhanced Raman scattering
SNPs	Single nucleotide polymorphisms
TSA	Tyramid signal amplification
TSDRs	Toehold-mediated DNA strand displacement reactions

Introduction

Deoxyribonucleic acid (DNA), as the storage of hereditary information, is one of the most studied biomolecules since its inception by Watson and Crick in 1953. DNA consists of two complementary strands comprising of four repetitive nucleotides which are assembled by hydrogen bonds and stacking interactions to form a double-stranded DNA (dsDNA) [1]. Nucleic acids, in particular DNA, play a vital role in biological studies and clinical diagnoses. In addition, nucleic acids can be employed as appropriate recognition elements for analyzing ions, small molecules, proteins, and even whole cells. Due to these intriguing ligand-binding properties, nucleic acids are used for fabricating different analytical devices such as point-of-care analyzers, nanocarriers, intelligent biosensors, and logic nanodevices [2–7]. In this respect, platforms exploiting the hybridization capability of nucleic acids for the detection of DNA/RNA have recently received increasing attention. One of the most important challenges related to these bioanalytical devices is the sensitivity issue in detecting low-abundance nucleic acid molecules. To improve limit of detection (LOD) value, which is a function of signal strength (or sensitivity), the hybridization assays have been integrated with different modern amplification strategies, including target-and-signal-based amplification methodologies (Scheme 1). In brief, target amplification is referred to any method which directly increases the copy number of target molecules, while signal amplification uses highly sensitive reporter molecules or probes to detect the target molecule (directly increase the signal) without increasing the amount of the target. These strategies can be performed in thermocycling or isothermal conditions [8]. In fact, to circumvent the limitation of thermocycling amplifications, various isothermal strategies have been developed [9]. In contrast to PCR, which is a typical example of thermocycling amplification technique (Scheme 1), isothermal amplification can be performed in a single-temperature incubation [10, 11].
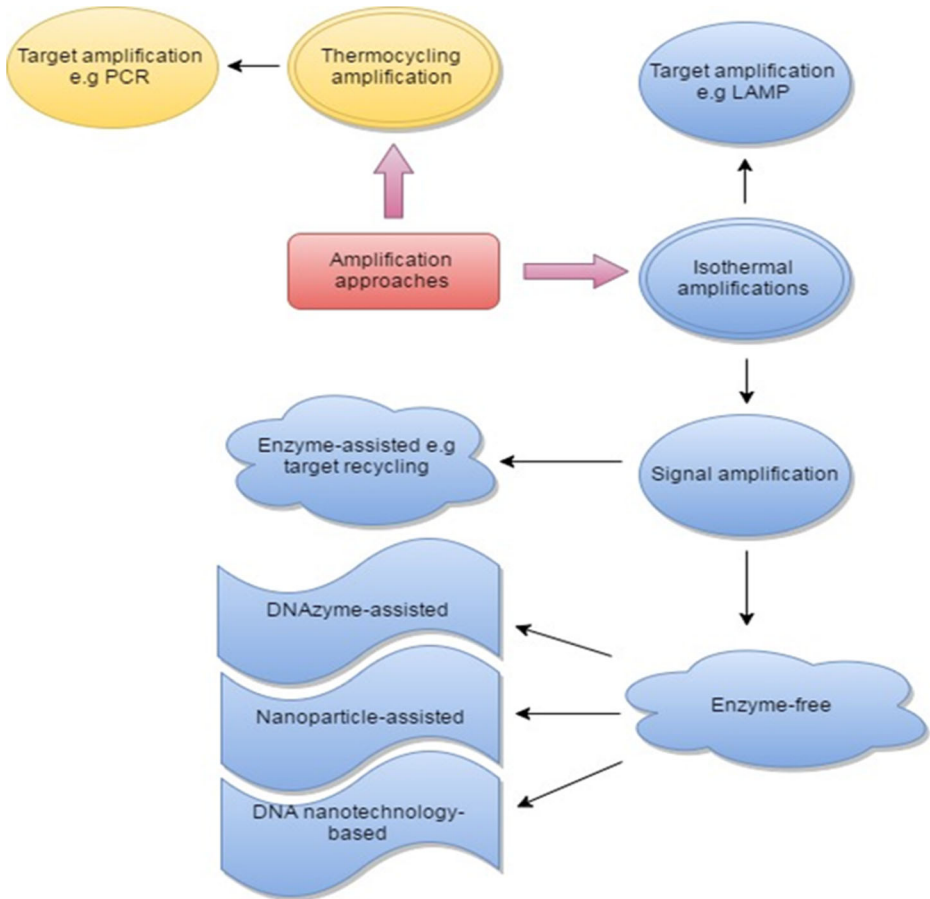
Scheme 1 Classification of nucleic acid amplification approaches. The amplification procedures can be classified into thermocycling and isothermal approaches

The signal amplification scheme in the isothermal context provides a simple and programmable tool for the detection of nucleic acids [12]. To compare the amplification efficiency of different signal amplification platforms, a quantitative value is defined according to the stoichiometry of target consumption to the number of signal generation (target/signal, t:s) [13]. For instance, in a method like amplification-free molecular beacon (MB) biosensing, there is a t:s value equivalent to X:X, which exhibits the generation of X signals in response to X target molecules. The previously developed signal amplification methods can be categorized into linear and exponential amplification platforms. Linear amplification strategies have a t:s value of X:nX (X target molecule/nX signals). Despite the advantages of linear amplification, the interrogation of low-abundance nucleic acids in solution has provided a high demand for designing more sensitive biosensing platforms. Advances in biotechnology, chemistry, and nanotechnology have led to the design of new exponential amplification strategies with a t:s value of X:expX. In addition, exponential amplifications can be performed under cascade reactions in order that the upstream products act as a trigger for the activation of a downstream signaling component [14]. Exponential amplification methodologies that are achieved via cascade reactions could overcome the limitation of linear and conventional exponential amplification strategies [15].

There are other reviews on the similar subject, like the one by Nie which has focused on bio-conjugated nanoparticles for in vitro applications and medical diagnoses [16]. In the present review, however, we describe those signal amplification-based methodologies for detecting nucleic acids that have paved the way for in vivo imaging. We firstly summarize different signal amplification-based detection strategies for nucleic acids in vitro, and subsequently extend the methods to in situ (e.g., cell lysates and fixed cells) and in vivo conditions.

Why Shifting Away from In vitro Detecting to In vivo Imaging

Detections in cell-free settings are carried out by analyzing biological samples such as blood, urine, and tissue in a test tube. These methods have attracted much attention due to their particular features in medical decisions and treatments [17]. First, in a test tube, all conditions (such as temperature, pH, molecular crowding, and kind of targets) can be precisely controlled, whereby it allows an easier design, implementation, and evaluation of the assay. Second, detections in the test tube are performed without any trouble regarding the reagent delivery and accessibility of the specific sequence on the target. Last, risk degradation by nucleases is minimized in cell-free settings, which lead to the reduction of chemical modification of oligonucleotide probes [18]. In short, simplicity, specificity, and convenience of in vitro diagnoses have caused these methods to continue to be of interest.

Despite their considerable advantages over other environments, in vitro assays suffer from certain limitations. In some cases, the results obtained from in vitro studies cannot be easily extrapolated to living cells. Two plausible justifications are possible for this inconsistency: (i) in a test tube, the bulk analyte is determined regardless of the differences in cell-to-cell variations and (ii) conditions of living cells are different from in vitro systems because the presence of crowding materials, nucleases, and secondary structures in the target molecule alters the capability of the assays [18]. To address these challenges, experimental studies are performed in fixed cells or in situ systems that provide a milieu close to the natural environments. These systems share some properties of in vivo conditions—such as the presence of complex matrixes, digestive nucleic acid enzymes (DNases and RNases), and nucleic acid-binding proteins—and also struggle with certain problems associated with in vivo imaging. The first challenge, which is related to the delivery and distribution of exogenous probes to the cell, is generally obviated by choosing an appropriate delivery system. The second problem is the inaccessibility of target nucleic acids, which occurs due to the presence of complex secondary structures in the target nucleic acids. This challenge is addressed by some pretreatments, such as heating and/or using chemical reagents like formaldehyde that fixes the cells by cross-linking interactions. In addition, the developed helper oligonucleotides will have a potential to be used for live-cell analysis of nucleic acids for unwinding the secondary structures of target molecules [19]. The third issue is the stability of exogenous DNA and RNA probes for in situ and in vivo analyses. Based on the recent studies, salt concentration, probe structures (single- or double-stranded DNA, or more complex DNA structures like DNA origamis and lattices), and the presence of chemically modified nucleotides in the probes can alter the stability of exogenous probes for in situ applications.

In situ conditions, by imitation of live-cell conditions, pave the way for adopting biosensing methods for in vivo analyses. However, due to particular pretreatments and washing steps, in situ approaches are laborious and time-consuming. Moreover, these techniques are based on chemical fixations of the cells, which put negative impacts on the accuracy of nucleic acid analyses [20]. The final goals in such methods are achieved when the stability, dynamics, functions, and interaction of nucleic acids can be easily investigated.

Different Signal Amplification Approaches

1 Enzyme-Assisted Methods The combination of protein enzymes with an appropriate recognition element is an efficient strategy to improve the assay sensitivity. Enzyme-assisted methodologies are performed under two main strategies: a direct conjugation of assay's components like oligonucleotides to enzymes (e.g., phosphatases or peroxidases) and implementation of the assays by polymerases and/or nucleases [21]. By applying enzymes, linear signal amplification strategies can be upgraded to exponential amplification reactions [22].

Enzyme-Assisted Approaches for In vitro Detections

Target recycling methods have provided appropriate strategies for in vitro detection of nucleic acids. In this process, releases of the target molecules from probe-target complexes are usually catalyzed by activity of either nucleases or DNA polymerases (Fig. 1) [25, 26]. Afterwards, the target molecules enter new signaling cycles and generate more detectable products. Many of these platforms use specific nucleases based on the substrate type, their mechanisms, and restriction sites. For example, the function of exonuclease III, compared to nicking endonucleases, is independent of its restriction site, while both act on the same substrate, dsDNA. The mechanisms of these nucleases are also different: nicking endonucleases just cleave one strand of the double strands on their restriction sites, while exonuclease III can digest nucleotides from blunt 3′-termini, nucleotide by nucleotide. Creating a nick on a double strand alone will not guarantee the target strands escaping; however, if this happens, an increase in the entropy of the system will mediate the target releasing. Selecting an appropriate cleavage site on the products increases the rate of target release and hence improves the efficiency of the signal amplification process. DNA polymerases are alternative enzymes for target recycling purposes. Some of these enzymes have exploited two different functions, including DNA polymerization capability and DNA strand displacement activity [24, 27]. However, inhibition of DNA polymerases by their product, pyrophosphate, is the main limitation of these enzymes. Exonuclease III is the most common enzyme of choice for target recycling strategy due to high speed in target processing and lack of constraints on sequence variation [28–30]. Ye et al. used exonuclease III-based signal amplification strategy with fluorescence polarization technology for discriminating single nucleotide polymorphism (SNP) [28]. Zhang and co-workers recently designed a bacterial DNA biosensor by implementing lysozyme signaling probe, magnetic beads, and exonuclease III for the detection of femtomolar concentrations of the target DNA [31].

Enzyme-Assisted Approaches for In situ Analyses

In situ hybridization (ISH) is one of the classical methods for analyzing nucleic acids, which was introduced by Gall et al. in 1969 [32]. ISH is an amplification-free platform with a t:s mode of X:X, the LOD value of which is not good enough for detecting low-abundance nucleic acids with non-radioactive signal read-out [23]. Despite the sensitivity of radioactive labels in original ISH, this type of signal element suffers from some inherent limitations such as hazardous to health, high prices, and poor spatial resolution of signal [33]. In this regard, a fluorescence-based measurement, known as FISH, was developed for overcoming these limitations. Exploiting the enzymatic amplifications and employing a large number of probes for enhancing sensitivity was the next step for this journey. Utilizing enzymes such as AP and PO as labels improves the
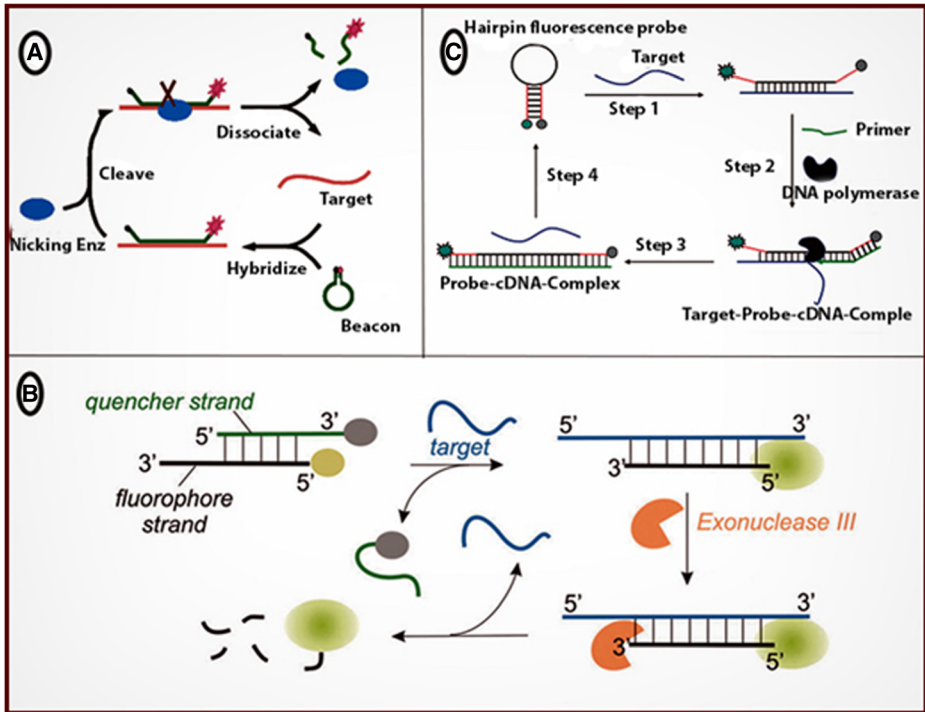
Fig. 1 Three representative examples of target recycling strategy. Release of the target molecules from probe-target complexes are catalyzed by the activity of enzymes (nucleases or polymerases). **a** A molecular beacon is hybridized to a target and provides an appropriate substrate for specific nicking enzyme. The nicking enzyme creates a nick on the probe (one strand of the double strands on the restriction site) so that its fluorophore and quencher are separated and fluorescence signal is generated [21]. **b** After the hybridization of the target to labeled probe, an appropriate substrate for exonuclease III is generated. Exonuclease III digests nucleotide from blunt 3′-end of the probe and generates fluorescent signals [23]. **c** Applying DNA polymerases with strand displacement capability causes the target recycling process to perform with improved efficiency [24]

sensitivity of original ISH. These enzymes can perform catalytic reactions on substrates, which leads to the deposition of chromogenic or fluorescent products over the site of reaction [34]. Following the researchers' attempts, a new approach, namely TSA, has been employed for improving the efficacy of enzyme-based ISH methods (Fig. 2) [33, 35, 36].

Enzyme-Assisted Approaches for In vivo Applications

Besides the aforementioned applications, enzyme-assisted strategies can be finitely employed for in vivo conditions. Min and co-workers demonstrated an enzyme-assisted signal amplification strategy for in vivo monitoring of microRNAs (miRNAs) (Fig. 3) [37]. They used a molecular detecting strategy by applying molecular probes which contain yellow aggregation-induced emission luminogens (AIEgens) with super photostable property. In comparison with conventional MB, the new probe does not need a quencher and shows much higher fluorescence intensity. In this system, exonuclease III was employed for improving the signal intensity. However, employing protein enzymes for live-cell imaging is associated with some weaknesses, such as low penetration to the cells due to their high molecular weights [38] and the presence of effective inhibitors in the intracellular milieu.
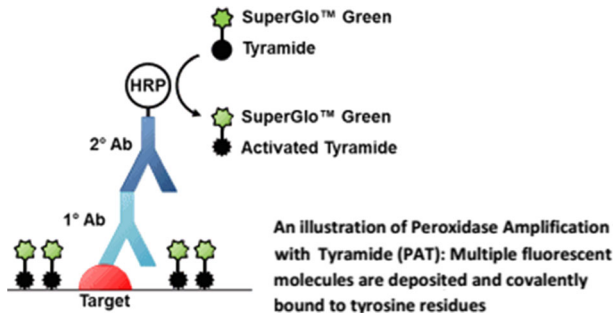
Fig. 2 Schematic representation of TSA mechanism. Fluorescently labeled tyramine in the presence of hydrogen peroxide is converted into a highly reactive oxidized species by peroxidase enzyme. The oxidized product can bind to tyrosine residue of proteins at or near the target site (SuperGlo™ products, online: http://fluorescent-solutions.com/)

2- Enzyme-Free Methods Attainment of high sensitivity by protein enzymes is undeniable; however, the properties of enzymes, such as high cost, specific storage, inhibition under cellular conditions, and special conditions for their activity have greatly limited their vast applications for in vivo nucleic acid imaging. Over the recent years, several enzyme-free signal amplification approaches have been developed to address the shortcomings of enzyme-based
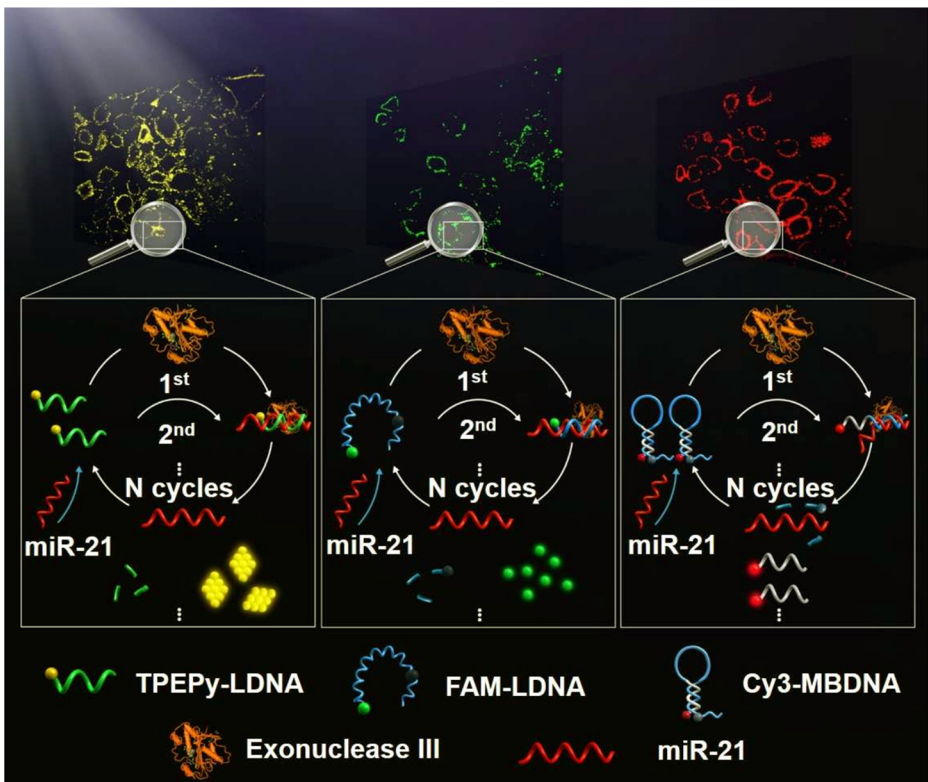

Fig. 3 Schematic representation of the target recycling strategy for in vivo imaging of miRNAs. Exonuclease III can recycle the target molecule by digesting the probe molecule. It improves the signal intensity of the system (adopted from [37])

methods. These biosensing technologies can be classified into DNAzyme-, nanoparticle-, and DNA nanotechnology-based platforms.

2–1 DNAzyme-Assisted Platforms DNAzymes, DNA molecules with catalytic activity, have been widely evaluated as recognition and catalytic units for enzyme-free methods due to significant properties such as simple synthesis, low cost, high stability, and ease of labeling [39–42]. DNAzymes can catalyze a diverse range of reactions, including nucleic acid cleavage [43–45], DNA ligation [46], peroxidation [44], and DNA phosphorylation [47] (Fig. 4).
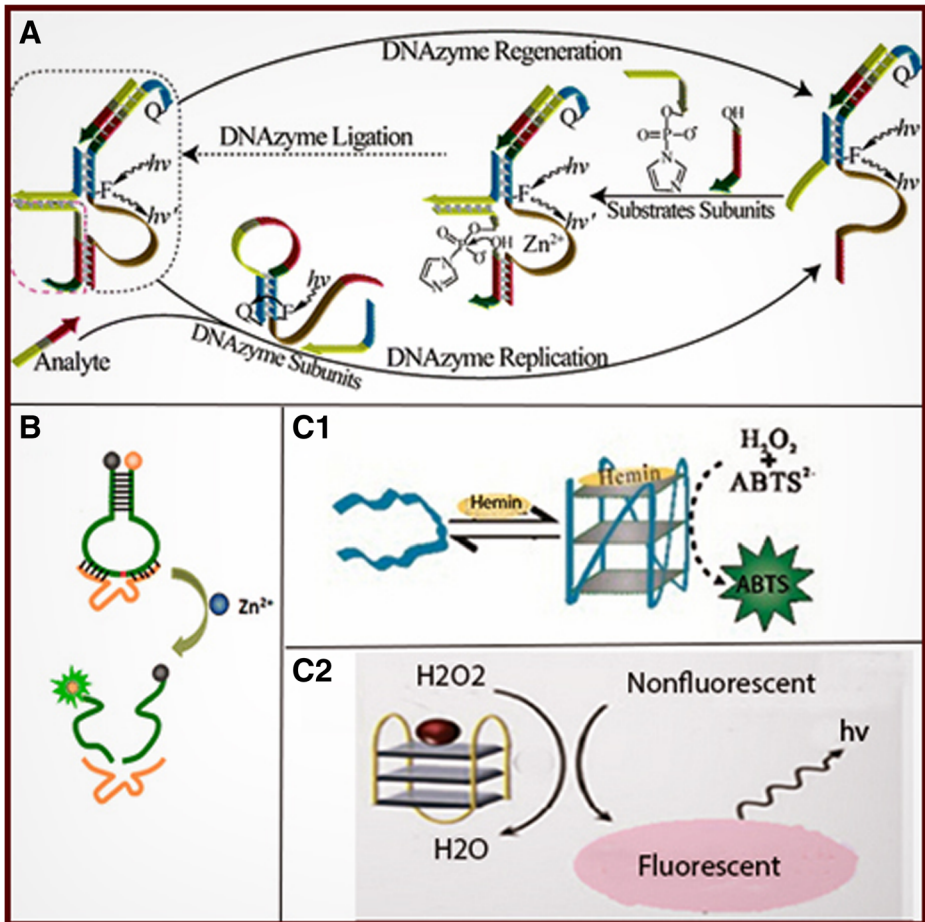

Fig. 4 Variation of DNAzyme-catalyzed reactions for biosensing applications. **a** A general mechanism for an RNA/DNA ligation reaction: the Zn^{2+} ligation DNAzyme can catalyze the formation of a new phosphodiester bond by the condensation of the 5′-hydroxyl of one oligonucleotide and a 3′-phosphorimidazolide on another oligonucleotide (adopted from [46]). **b** DNAzymes for nucleic acid cleavage. The digestion process is mostly mediated by a metal ion-dependent interaction in DNAzyme molecule (adopted from [48]). **c** Peroxidase-mimicking DNAzyme, which is formed through the interaction of hemin with guanine-rich sequences (G-quadruplex). An increase in peroxidase activity of hemin by G-quadruplex structure can be observed by appropriate peroxidase substrates such as ABTS (C_1) and luminol (C_2) for colorimetric and chemiluminescent assays, respectively (adopted from [49])

DNAzyme-Assisted Approaches for In vitro Detections

Since the enzymatic activity of DNAzymes has been demonstrated, many platforms have been designed for the efficient detection of a wide range of biomolecules [50]. For instance, Sando et al. exploited phosphodiester bond hydrolysis activity of a chimeric DNAzyme (deoxyribozyme with a few ribonucleotide monomers) for developing a signal amplification assay [43]. In this sensing system, the active structure of the DNAzyme is formed only when the target substrate is presented. By hydrolytic self-cleaving activity of the chimeric DNAzyme, its fluorophore/quencher pair is separated and fluorescent signal is detected. Another prototype example of DNAzymes is hemin/G-quadruplex complex that exhibits a peroxidase-like activity. This peroxidase-mimicking DNAzyme has been used for developing different colorimetric [44, 51], electrocatalytic [52], and chemiluminescent [53] biosensing platforms for the analysis of nucleic acids [44], metal ions [54], and proteins [55]. Hemin/G-quadruplex complex can catalyze the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid (ABTS)$^{2-}$ and luminol in the presence of hydrogen peroxide to generate colored product ABTS$^{\bullet-}$ and a chemiluminescent product, respectively [56, 57]. On the other hand, ligation DNAzymes can catalyze the formation of a new phosphodiester bond between two oligonucleotides. In principle, the analyte DNA can activate the ligation DNAzyme and the product can be used for signal amplification [46, 58]. In addition, combination of DNAzyme with other methods, such as gold nanoparticle (AuNP)-based signal amplification, toehold-mediated rolling circle amplification, and catalytic hairpin assembly (CHA) improves the sensitivity of DNAzyme-assisted methods [59–61]. DNAzymes have also been integrated with fluorescent-based methodologies. In these platforms, due to the incomplete fluorescence quenching of organic molecules, quenching materials such as graphene oxide (GO) and AuNPs have been exploited in the development of new DNAzyme-based fluorescent sensors [53, 62].

DNAzyme-Assisted Approaches for In situ Analyses

DNAzymes have been extensively used for the development of highly sensitive and specific detection platforms for metal ions [63], small molecules [64], and biomolecules [65]. For instance, Pavlov et al. used peroxidase-mimicking activity of the hemin/G-quadruplex DNAzyme for the detection of DNA and low molecular weight analytes in in situ conditions [66].

DNAzyme-Assisted Approaches for In vivo Applications

To the best of our knowledge, most applications of DNAzymes for in vivo experiments pertain to the therapeutic purposes [67]. However, metal ions [68, 69], proteins [41], and nucleic acids [70] have so far been analyzed in intercellular mediums by DNAzyme-assisted platforms. Zhang et al. recently designed a programmable nanodevice based on MANzyme—a type of DNAzyme that splits into two partzymes for intracellular imaging and logic gated-drug delivery [70]. In the presence of target miRNAs, partzymes get close to each other and make an active MANzyme capable of cleavage of a fluorescently labeled oligonucleotide. The signal amplification would happen by autonomously moving of the active MANzyme to the neighboring substrates for the next cleavage. MANzyme also serves as a gatekeeper in order to retain doxorubicin, an anti-cancer drug, inside its nanocarrier.

2–2 Nanoparticle-Assisted Methods The increasing demand for the use of nanoparticles in diagnostic applications is related to their unique properties, such as high surface area to volume

ratio, strong signal intensities, stability, biocompatibility, and finely tunable surface chemical properties [17, 71, 72]. Various nanomaterials, such as metallic nanoparticles, quantum dots (QDs), magnetic nanoparticles, liposomes, carbon nanotubes, and silica nanowires have been employed for the detection of biomarkers [73–80]. In addition to their general properties, nanoparticles exhibit distinctive characteristics, such as bright and multicolor-tunable fluorescence emission and LSPR effect which have made them suitable for diagnostic utilizations. QDs are a good example of "fluorescent nanoparticles" and AuNPs are a popular "plasmonic nanoparticle" [81, 82]. In addition, magnetic nanoparticles have attracted increasing attention for utility in signal amplification technologies [80].

Nanoparticle-Assisted Methods for In vitro Detections

Bio-Barcode Based Methods Bio-barcode assay was demonstrated for the first time by the Mirkin group for the detection of nucleic acid and protein targets with attomolar sensitivity [83]. The bio-barcode amplification assay is composed of AuNPs covalently labeled with a dense shell of short oligonucleotides, known as barcodes, which are complementary to a target nucleic acid strands. A magnetic microparticle (MMP) with recognition elements complementary to a specific region of the target molecule and barcode-AuNP is the main ingredient of the platform. The particles in the presence of the target nucleic acids form a sandwich structure that is amenable to harvest by a magnetic field. The collected sandwiches release their barcodes which are recognized by scanometric assay under chemical treatments (Fig. 5) [84].

Remarkable stabilities against environmental impacts and substantial sensitivity due to employing hundreds of barcodes on the nanoparticle surface are the exclusive features of this method. Dong et al. used bio-barcode assay and enzymatic target recycling strategy for the detection of microRNAs [85]. In this strategy, the barcode oligonucleotides enter a target recycling stage after bio-barcode dissociation step. This dual signal amplification strategy provides an LOD value remarkably lower than the conventional bio-barcode assay (Fig. 6a). Despite the high sensitivity of this method, it suffers from some nontrivial weaknesses. In this process, barcode dissociation step requires a long incubation time with toxic chemical reagents [87]. Thus, some researchers have modified the principle of the bio-barcode assay, whereby it does not need the separation step [86, 88–90]. For example, Meng and co-workers used bio-barcode strategy besides DNAzyme signal amplification for the electrochemical detection of microRNAs [89]. Chen and co-workers reported a new electrochemiluminescence-based bio-barcode assay for the detection of tumor cells [90]. Cui's group used hairpin bio-barcode assay in conjugation with an electrochemical detector for analyzing target DNA molecules [86] (Fig. 6b).

Liposome-Assisted Methods Liposomes are artificial microscopic vesicles made of the lipid bilayer, which have been applied for different purposes, such as drug and gene delivery and biosensing applications [91]. Due to their unique properties, such as biocompatibility, high surface area, large internal volume, and modifiable surface chemistry, liposomes have offered special opportunities for utility in signal amplifications. In fact, liposomes can be directed to the targets by their specific surface-confined bio-elements and then execute their functions by escaping the encapsulation signal markers such as dyes, enzymes, and nucleic acids in pre-defined regions (Fig. 7). Based on the type of signal markers, different biosensors including colorimetry, chemiluminescence, and electrochemical [92] have been fabricated. In this respect, Chumbimuni-Torreset and co-workers demonstrated an electrochemical signal amplification strategy using
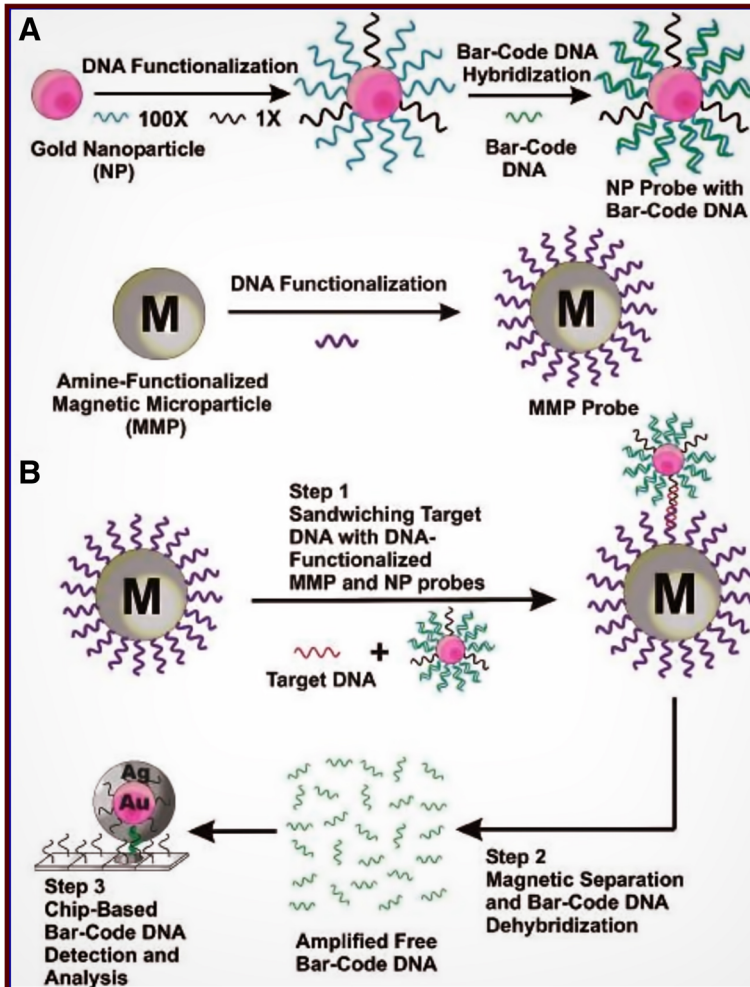
Fig. 5 Principle of bio-barcode assay in the original approach. *A* AuNPs covalently labeled with a dense shell of barcodes. *B* The particles form sandwich complexes in the presence of the target nucleic acids. This complex can be separated by a magnetic field and then the barcodes are released and recognized by scanometric assay [83]

ion-loaded liposome nanocarriers [93]. In the presence of the target, a sandwich hybridization assay has led to the capture of the ion-loaded liposomes on the surface of potentiometric electrode and then the release of the signaling ions by a surfactant. In another work, a dual amplification strategy for a highly sensitive detection of DNA was reported by Zhou and co-worker [94]. In this strategy, DNA probes complementary to a target DNA were dual-labeled with MMP and dye-loaded liposome at their 3′- and 5′-ends. Upon hybridization of the target to the probe, a double-stranded DNA with a binding site for exonuclease III emerges. The exonuclease triggers an enzymatic target recycling process, whereby a fluorescein-encapsulated liposome is released. Upon harvesting and disrupting the liposomes, the fluorescent intensity of the solution increases consistently with the target DNA concentrations. In addition, Bui and co-workers used liposome-assisted immunoassay for visual detection of live pathogen of *Salmonella* [95].
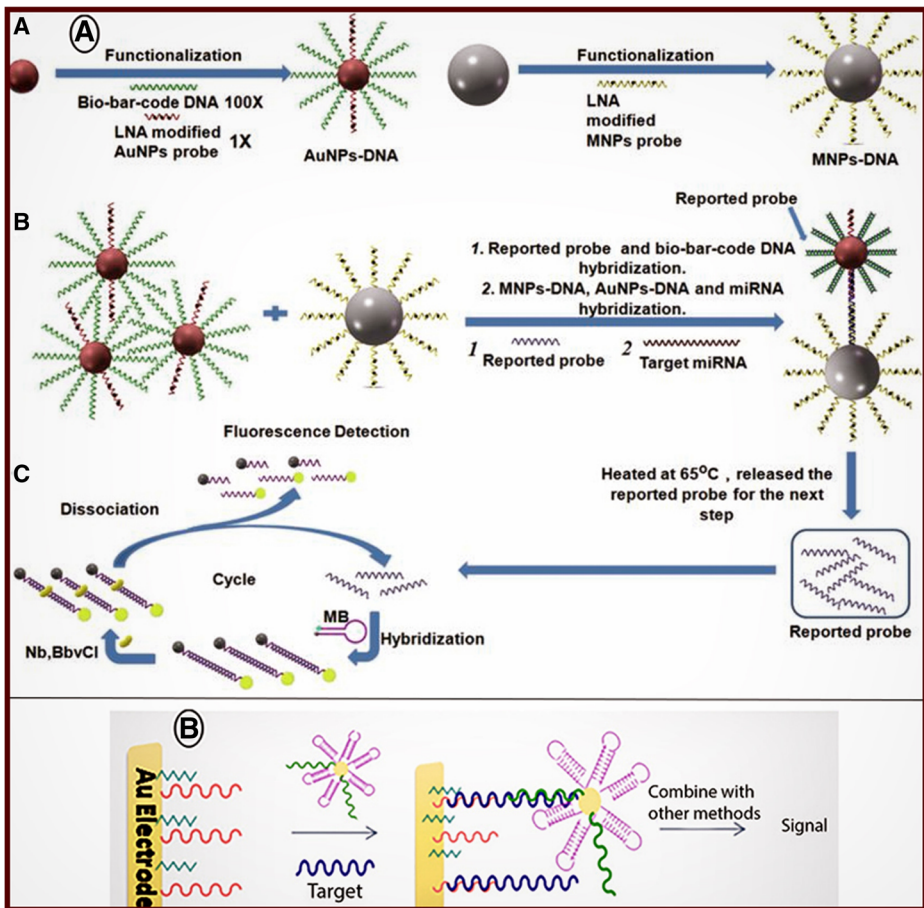
Fig. 6 Different strategies for the application of bio-barcode assay. **a** Change in the original approach by replacing the scanometric DNA detection with molecular beacon-based analysis [85]. **b** Modification of the bio-barcode assay with bi-functionalized AuNP for an electrochemical approach. AuNPs are labeled with two types of oligonucleotides (stem-loop and capture oligonucleotides) instead of barcodes (adopted from [86])

Other Nanoparticle-Based Methods Besides AuNPs and liposomes, silver nanostructures [96], QDs, GO, and specific polymers have been used for optical detection of nucleic acids. Chen et al. designed a visual detection platform, known as "sticky balls," through the linking MMPs, PMPs, and AuNPs together by a target nucleic acid. After being harvested by magnetic field, a turbid cloudy pellet appears which can be evaluated by the naked eyes and is without amplification [87]. Dong and co-workers reported a novel biosensing platform based on fluorescence resonance energy transfer (FRET) between DNA hairpin-functionalized QDs and GO [97]. In this strategy, a MB was developed based on fluorescence property of the QD and quenching capability of the GO. Li et al. developed a new method based on the use of DNA capture probe-labeled MMP, oligonucleotide-tagged CuO nanoparticle (oligo-CuONP), fluorescent DNA–Cu/Ag NC, and 3-mercaptopropionicacid (MPA) for miRNAs analysis [98]. In the presence of target
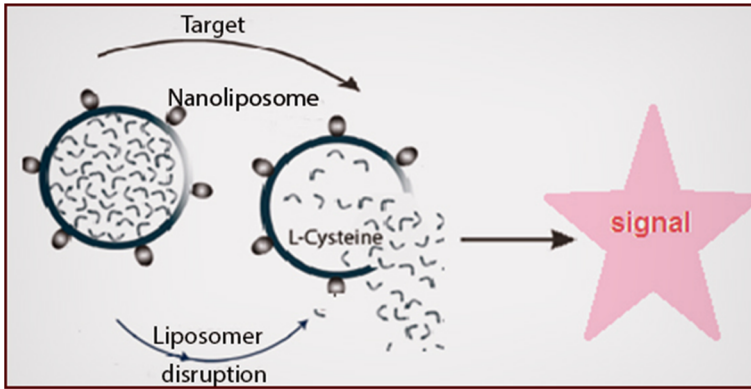
Fig. 7 Principle of liposome-based biosensing. Upon hybridization of the oligonucleotide-labeled liposome to the target and lipid bilayer disruption, a specific signal generating marker is released (adopted from [86])

miRNA, a sandwich complex composed of MMP probe/miRNA/oligo-CuONP was formed in solution and then separated by means of magnetic field. The harvested complex was dissolved by an acid, whereby the CuONP was converted into copper (II) ions (Cu^{2+}). The fluorescence emission of the DNA–Cu/Ag NCs increases upon the increasing concentration of Cu^{2+}, which is directly proportional to the amount of target miRNA (Fig. 8).
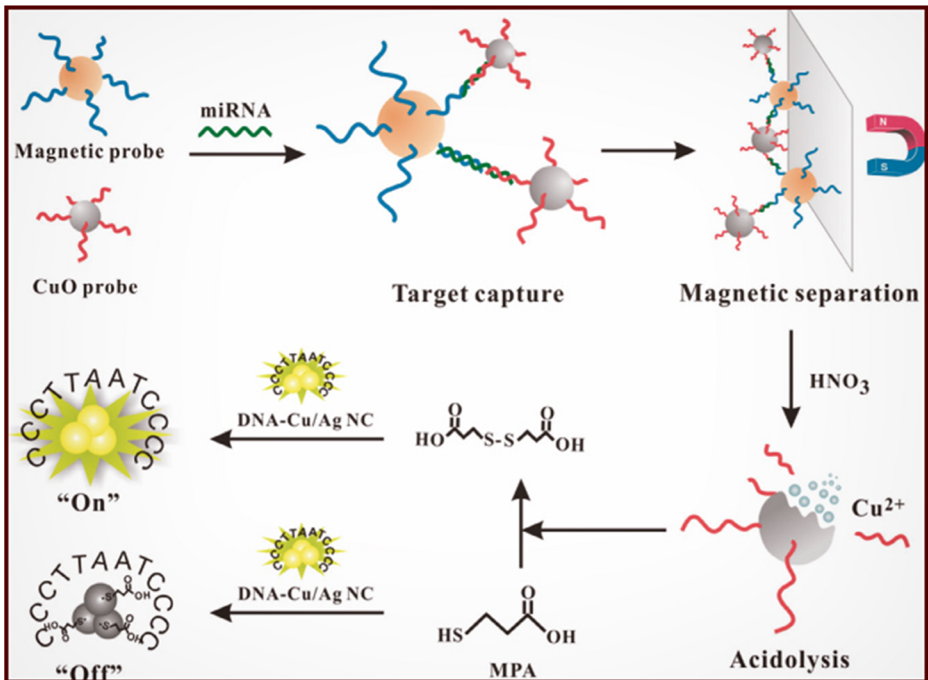

Fig. 8 Illustration of the proposed method based on CuO nanoparticle. The resultant sandwich complex (MMP–miRNA–CuONP) is formed in the presence of the target miRNA. After separation of the sandwich complex by a magnetic field, CuO nanoparticles are dissolved by acidolysis reaction in order to convert CuO nanoparticles into copper (II) ions. MPA molecules are oxidized by copper ions and the products keep the fluorescence emission of the DNA–Cu/Ag NCs turned on [93]

In recent years, to improve the sensitivity of nucleic acid biosensing methods, nanoparticle-assisted methods have been equipped with other methods like enzymatic signal amplification strategies [99–101]. In this respect, Degliangeli's group coupled enzymatic target recycling method with DNA–AuNP for the direct detection of miRNAs [100]. In the presence of target miRNA, the produced DNA–RNA complex became substrate for the endonuclease DSN—DSN cleaves DNA in a heteroduplex nucleic acid—thereby generating fluorescence signals by releasing the fluorophores away from the gold surface. Employing AuNPs in lateral flow biosensor is another method for nucleic acid detection [102–104]. This method is based on the aggregation of AuNP-labeled probe on the test zone of the biosensor.

Nanoparticle-Assisted Methods for In situ Analyses

With the rapid development in nanotechnology, various nanoparticles are employed as excellent biological tags for in situ analysis of nucleic acids. For example, Wu and co-workers reported a one-step nanoparticle-based strategy containing the MB for analyzing exosome-entrapped RNA molecules [105]. In this strategy, hybridization of the MB probes to target RNA is mediated by the fusion of the entrapped MBs in cationic lipoplex nanoparticles to RNA-containing exosomes. It causes the sensitivity of the assay to significantly improve over the original MB-based assays. Cui et al. developed a method based on the application of GO for miRNA detection in complex biological samples [106]. In fact, they used the fluorescent quenching property of GO for simultaneous analysis of multiple miRNA targets.

Nanoparticle-Assisted Strategies for In vivo Applications

Nanoflares Developed by Mirkin group, nanoflares are polyvalent nanostructures consisting of a spherical AuNP covalently functionalized with a shell of oligonucleotides [107]. This new nanostructure-based technology has exhibited significant advantages over other biosensing nanodevices, such as low toxicity, high stability against enzymatic degradation, and reasonable efficiency in cell delivery. When target messenger RNA (mRNA) binds the recognition sequence, the reporter flare strand is released by a toehold-mediated strand displacement, providing a fluorescent signal (Fig. 9a). In fact, the role of AuNPs is fundamental in this method since (i) they improve the stability of confined oligonucleotides by inhibiting enzymatic degradation through the attraction of high salt concentrations around the oligonucleotide shell, (ii) they play a quenching role for flares, and (iii) they serve as a transfection system with a low toxicity [110]. Due to its interesting properties, the nanoflare technology has been used for detecting a variety of biomolecules, such as small molecules, proteins, and nucleic acids in living cells [111]. Based on this technology, Prigodich et al. designed a multiplex nanoflare for the detection of mRNA targets capable of normalizing the cell-to-cell target variations [108]. Halo and co-workers reported an antibody-functionalized nanoflare for detecting circulating tumor cells in blood by flow cytometry. Yong et al. demonstrated a FRET nanoflare to enhance the sensitivity of the assay by reducing the background signal [109]. In this strategy, a double-labeled fluorescent reporter flare, which is displaced by the target mRNA molecule, forms a stem-loop structure. The hairpin structure brings the donor and acceptor fluorophores together in order to generate a FRET signal (Fig. 9b). Shi's group used a fluorescence lifetime imaging microscopy instead of fluorescence microscopy for increasing the sensitivity of the nanoflare biosensings [110].
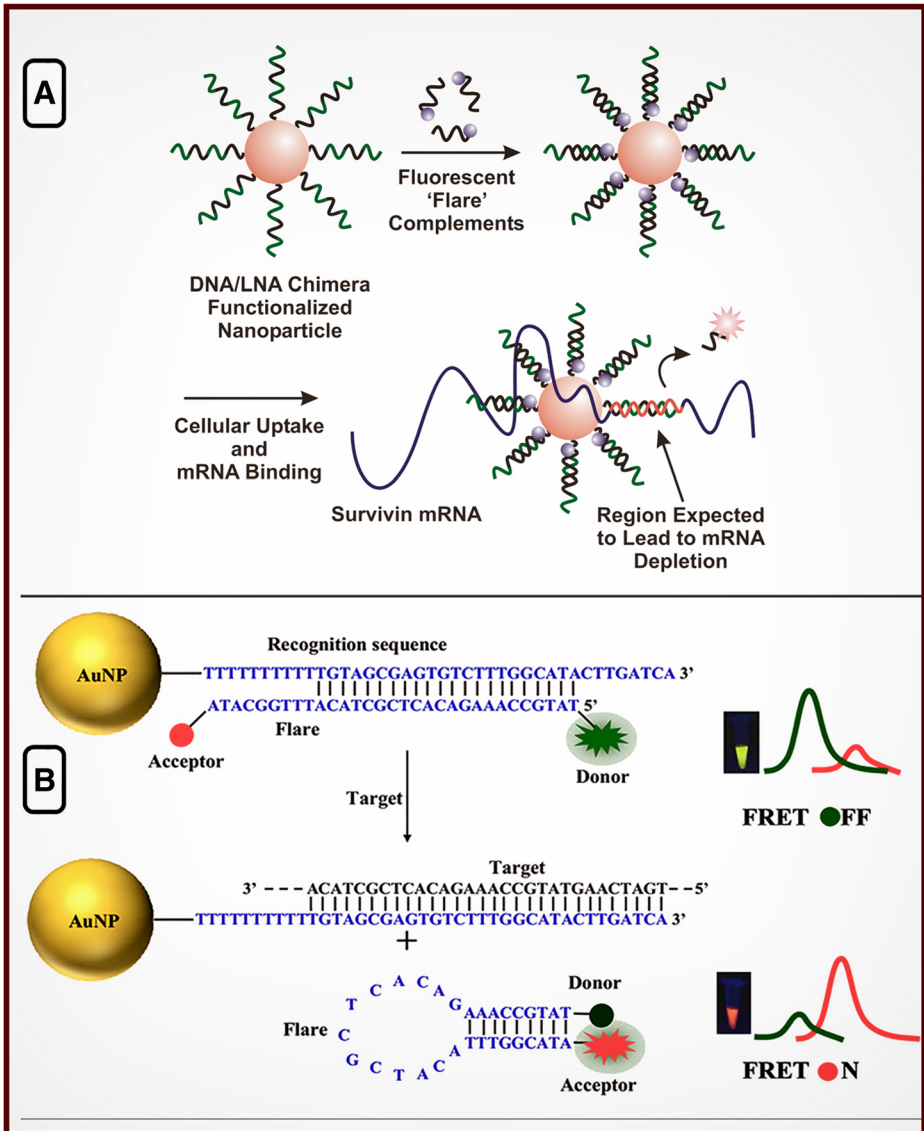
Fig. 9 Schematic representation of the nanoflare technology. **a** AuNPs are first functionalized with a shell of single-stranded DNA monolayer, and then the flares are hybridized to confined DNA strands. The flare probe is displaced from dsDNA structure by a target mRNA molecule through the toehold-mediated strand displacement reaction and generates fluorescent signals [108]. **b** FRET-based nanoflare [109]

QD-Based Methods The application of QDs for in vivo imaging is limited by their high cytotoxicity effects on different cell lines. However, due to high fluorescence quantum yields, researchers attempt to improve the biocompatibility of these nanostructures. By eliminating cadmium (Cd) and producing Cd-free QDs, like silicon (Si)-QDs, the biocompatibility of the QDs has been significantly improved [112]. In addition, these nanostructures have exhibited a remarkable resistance to photobleaching, which enhances the stability of fluorescent emission

intensity against cellular environment. For instance, Arap et al. reported a Si-QD method for cancer diagnosis [113]. In fact, this QD was used in the same format of nanoflares, which deployed QD-labeled flares instead of organic fluorophore-based flares.

2–3 DNA Nanotechnology-Based Devices While the attempts for the achievement of inexpensive, simple, sensitive, and specific new detecting strategies are continuing, DNA nanotechnology has opened a new horizon to detection methods. DNA nanotechnology has led to the design of simple and logical biosensing platforms through the elimination of extra reagents, like enzymes and complex steps. Moreover, due to the price reduction in DNA synthesis and enhancing signal amplification without using enzymes, this strategy has begun to receive more attention in recent years [114]. Advances in DNA nanotechnology by employing metastable DNA building blocks allow to construct different nanostructures by self-assembly reactions with static and dynamic features [115]. Generally, DNA nanotechnology is classified based on the reaction mechanism for product formation into two overlapping subfields, namely structural (static) DNA nanotechnology and dynamic DNA nanotechnology. Structural DNA nanotechnology attempts to build DNA nanostructures with desired conformation that settle in global thermodynamic equilibrium states, while dynamic DNA nanotechnology focuses on synthesizing DNA complexes with useful non-equilibrium behaviors [116].

DNA Nanotechnology for In vitro Detections

Employing DNA as "structural engineering material" has begun since 1982 by the seminal work of N. Seeman [117]. The field has been expanded considerably in recent decades to molecular biology for diagnostic and therapeutic applications. DNA self-assembly can be used for the construction of bulk-scale nanostructures, among the most common classes of which are DNA origami and hydrogels. However, these strategies can be combined with other methods as a result of signal amplification modularity. DNA origamis are created by folding a long single "scaffold" strand with a large number of "staple" strands (Fig. 10a) [18, 118, 121, 122]. In most cases, DNA origamis, which involve two- and three-dimensional structures, can be used as nanodevices for drug delivery, Boolean logic operators, and biosensors [123]. For the first time, in 2010, three-dimensional DNA origami structures were used for electrochemical analysis of SNPs by Pie et al. [119]. The authors accounted for the potential advantages of three-dimensional DNA nanostructures in the detection process and revealed that these DNA nanostructures had a high stability in biological fluids. Liu and co-workers developed a biosensing platform for the detection of lung cancer-related microRNAs [124]. In another research, by the integration of DNA origami with metallic nanoparticles, a powerful tool was developed for enhancing the yield of SERS-related assembly [121, 122]. In addition to metallic nanoparticles, DNA origami was used for the organization of semiconductors, nanocrystals, and organic chromophores [121]. Most of these platforms have the potential for utility in in vivo imaging studies.

DNA-based hydrogels have been exploited for detection purposes [120, 125]. These smart structures can respond to different stimuli, such as temperature, pH, and biomolecules, by a sol/gel phase transformation. This transition in physical behavior can be used in developing intelligent biosensors for nucleic acids analysis [126]. Helwa and co-workers used DNA aptamer as cross-linking agents for polymerizing linear polyacrylamide to a polymeric hydrogel for nucleic acid biosensing [127]. Applying DNAzymes for the fabrication of hydrogels
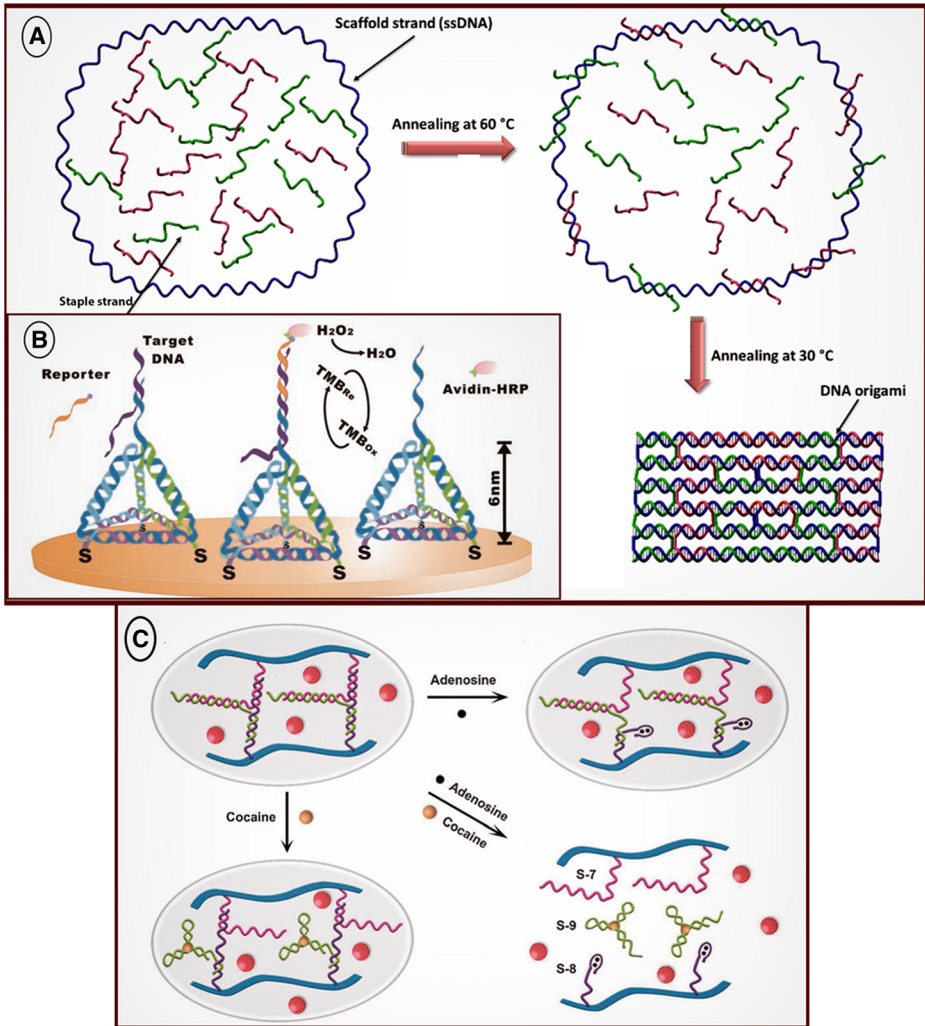
Fig. 10 a Fabrication of DNA origami nanostructures from scaffold and staple single-stranded DNA molecules [118]. **b** Tetrahedral DNA nanostructure-based electrochemical biosensor for the detection of nucleic acids [119]. **c** Schematic representation of DNA hydrogels. DNA hydrogens can transit from sol to gel or vice versa in response to target analyte [120]

has recently been initiated, but for the analysis of metal ions only [128]. Several recent studies have used nanoparticles as the signal indicator for hydrogel-based biosensing [126, 129]; however, attainment of high sensitivity needs to employ other materials, such as enzymes and DNAzymes along with nanoparticles [130]. Despite the advantages of DNA-based hydrogels, such as biocompatibility, flexibility, and ease of fabrication, employing these structures for in vivo analysis of nucleic acids is associated with some drawbacks. In fact, the interaction of bulk structure of hydrogels with complicated cell matrix has restricted the movement of the hydrogel to a specific cell region or compartment.

The first attempt for semi-programmable self-assembly of nucleic acids was launched by branched DNA (bDNA) assay [131]. In the basic format, bDNA assay is performed by

simultaneously binding a target nucleic acid to a capture probe and to an enzyme-conjugated oligonucleotide in order to produce a branched DNA structure on a solid surface (Fig. 11a). After washing the complex and adding the enzyme substrate, a visual signal is generated. bDNA assay has received the FDA approval for HIV1 and HCV load testing [132]. The field of programmable self-assembly of nucleic acids and, therefore, dynamic DNA nanotechnology has been revolutionized by introducing toehold-mediated nucleic acid strand displacement reactions (TSDRs). In TSDR, a nucleic acid toehold domain—a single-stranded nucleic acid contains between 4 and 8 nucleotides—is hybridized to its complementary domain and then by a branch migration process, the invader strand replaces an incumbent strand in a DNA duplex (Fig. 11b). In recent years, TSDRs have been used for developing intelligent biosensing platforms and biomolecular logic devices. It provides significant advantages over enzyme-assisted methods from two main aspects: Firstly, it needs only short metastable DNA motifs as "fuel" which can be assembled without requiring enzymes. Secondly, TSDRs are a dynamic process, which do not need external energy sources for their operation. The TSDRs drive by internal energy, which is stored in metastable motifs in the form of thermodynamic functions like change in entropy and enthalpy of DNA hybridization events. TSDR-based amplification methods can be classified into hybridization chain reactions (HCR), entropy-driven catalysis (EDC), and CHA reactions.

Hybridization Chain Reactions HCR is one of the main TSDR-based signal amplification strategies that is used for biosensing nucleic acids, proteins, metal ions, and even whole cells [8, 133]. According to the design of Dirks and Pierce [134], target molecules trigger a TSDR and open metastable H1 hairpin motif (Fig. 12a). Target–H1 complex is assembled with metastable H2 monomer by the same reaction, and a repetitive assembly process propagates until all hairpins in the reaction are exhausted. HCR constructs a long DNA concatemer with nicked and repetitive units, which can be easily analyzed by gel electrophoresis. In the basic format, the HCR products need no specific labeling process for their detection. In fact, by

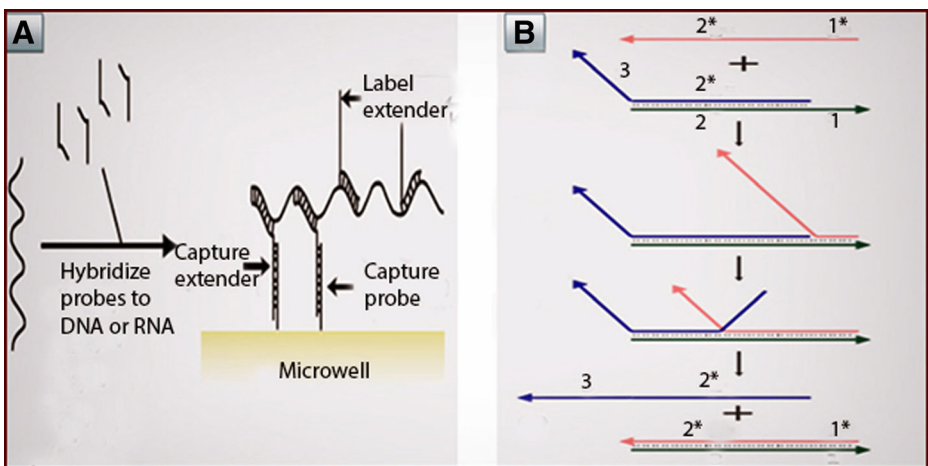Fig. 11 a bDNA assay, the assay is composed of a surface-confined capture oligonucleotide and enzyme- or dye-labeled oligonucleotide. In the presence of target nucleic acids, a sandwich complex is formed which produces a visual signal [132]. b The mechanism of toehold-mediated strand displacement: a toehold domain (1) is hybridized to its complementary domain (1*) and then a DNA strand displacement occurs between domains 2 and 2*
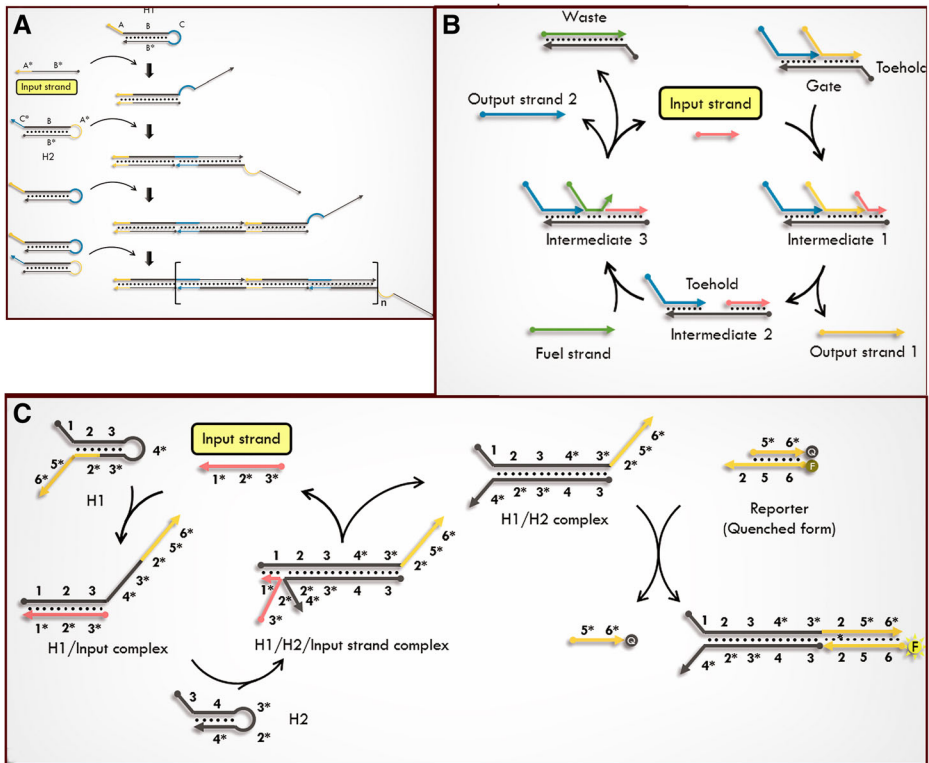
Fig. 12 Schematic representation of the reaction mechanisms of **a** HCR; an input strand or target can open H1 hairpin and generate H1–target complex. This complex can be hybridized with second hairpin (H2) and begin a chain reaction to create a long-nicked DNA duplex (concatamer) structure. **b** EDC; in this strategy, a specific input strand can release reside DNA strands on a duplex DNA by an increase in the entropy of the system. Exchanging the input target by fuel molecule results in the target recycling. **c** CHA; target or input strands can hybridize with their complementary domain in H1; then H2 hybridizes with its complementary domains on H1–target complex and releases the target molecule [135]

keeping constant the hairpin concentration, molecular weight of concatemers is controlled merely by the amount of target nucleic acids in the solution. High amounts of the target molecule are likely to produce DNA concatemers with a low molecular weight, while low amounts of it lead to the production of a low number of DNA concatemers with high molecular weights.

Though not without its advantages, HCR suffers from ceratin limitations, such as low sensitivity and low catalytic rate for the formation of the DNA concatemers. In this regard, continuous efforts during the past two decades have made it possible to develop improved biosensing strategies based on the basic HCR method. Employing enzymes, DNAzymes, fluorescent dyes, nanoparticles, and quantum dots as signaling labels have significantly improved the sensitivity of the HCR-based assays [116]. Based on fluorescent signal read-out, Li et al. implemented a MB-based platform for the detection of DNA [136]. Huang and co-workers used pyrene excimer for decreasing the LOD value of HCR-based assay in complex biological samples [137]. Moreover, Ellington et al. combined HCR with CHA in order to increase the sensitivity of HCR assay [138]. HCR was also implemented with DNAzymes that were conditionally activated by target molecules to form HCR concatemers

[139]. Yang and co-workers used the inherent sensitivity of plasmonic silver nanoprism in an enzyme-linked strategy for enhancing the sensitivity of HCR assay [140]. HCR on a solid surface was studied by Huang et al., which showed that the formation of DNA concatemers in solution is more favorable than that in solid-surface states [141].

Entropy-Driven Catalysis Designed by Zhang et al. [142], EDC can serve as an enzyme-free target recycling platform for the development of signal amplification techniques. In EDC, a specific input DNA strand can function as the catalyst for the generation of a plenty of output DNA strands. In the original method, the exchange of DNA strands begins in the presence of target molecules and pre-designed DNA motifs as substrate and fuel molecules. The target molecule initiates a cascade of TSDRs (Fig. 12b) so that a series of waste products are exhausted at the end from the reaction. In fact, EDC reaction proceeds through an increase in the net entropy within the system.

After the description of the reaction principles by Zhang et al., other researchers tried to improve the efficiency of the basic platform for developing more sensitive assays [135]. For example, Eckhoff et al. implemented EDC strategy with peroxidase-mimicking DNAzyme for a colorimetric analysis of nucleic acids [143]. Lv et al. improved this strategy for discriminating single nucleotide polymorphisms [144]. Based on the EDC strategy, Ravan implemented a two-layer feed-forward catalytic DNA circuit in conjugation with peroxidase-mimicking DNAzyme for the sensitive discrimination of single nucleotide substitutions [145]. Zong et al. improved the sensitivity of the EDC strategy by replacing the dsDNA fuel motifs in the original EDC with DNA hairpins [146]. Recently, Chen and co-workers have designed basic Boolean logic operations for the detection of nucleic acids based on the EDC and rolling circle amplification strategies [147].

Catalytic Hairpin Assembly This strategy can be defined as an enzyme-free target recycling method capable of periodically catalyzing the release of target nucleic acids during the reaction process [148] (Fig. 12c). According to Pierce and Yan's design, the metastable DNA hairpin H1 is first opened by target strand S1. The S1–H1 complex binds to a second hairpin (H2) that allows the target strand to be released from the S1–H1 complex [149]. The released S1 strands can trigger a new cycle of hairpin consumption. In this strategy, a reduction in the net enthalpy of the system, due to the formation of more base pairs in the products, has led to the reaction thermodynamically consume more hairpin monomers. In recent years, to improve the efficiency of the CHA, a new design strategy has been developed by Ellington's group to suppress the background assembly (system leakage) [150, 151]. They eliminated the "clamping" domains and extended the length of interacting domains for achieving a noise-free system with a good turnover catalytic efficiency and a shorter assay time. Chen et al. designed a new strategy by cascading several CHA reactions to enhance the t:s value of the assay to X:expX mode [152]. Huang et al. applied CHA reaction with two molecular beacon probes (MB1 and MB2) for the detection of DNA molecules [136]. The target DNA is hybridized to MB1 and turns on fluorescence emission. Afterwards, the MB2 is bound to MB1–target complex and releases the target DNA, which recycles for the next round of signal generation. According to their work, using two different MBs made the signal-to-background ratio to improve. Due to the target recycling property, CHA is able to combine with other strategies in order to decrease the LOD value of the assays.

DNA Nanotechnology for In situ Analyses

DNA origami, owing to its specific properties such as stability, programmability, and robustness in design, is a suitable choice for in situ applications. Tay et al. designed a DNA nanosensor inspired from snail for real-time in situ detection of mRNA molecules [153]. This nanosensor was composed of a sensory MB module and a DNA nanoshell that mimics the functional anatomy of a snail. In this platform, MB plays a recognizing role in the nanosensor and nanoshell facilitates cellular entry of the nanosensor and protects it from digestion. Pierce et al. used a programmable and sequence-specific HCR assay for in situ and multiplex analysis of mRNA targets [154, 155]. In contrast to other methods, HCR exhibits a high signal-to-background ratio, sharp signal localization, and deep sample penetration [154]. Haung and co-workers employed pyrene excimer, which has a stable and strong fluorescence emission intensity, to increase the sensitivity of HCR methodology for in situ studies [137]. In addition, Yamaguchi et al. improved this strategy by modifying the hybridization buffer and amplification protocol for reducing the assay time [38]. Recently, Huang and co-workers developed a FRET-based HCR strategy for in situ visualization of tumor-related mRNA [156].

DNA Nanotechnology for In vivo Applications

Generally, the probes and labels must meet several requirements for use in in vivo imaging. Firstly, the probes must be biocompatible and have minimal cytotoxicity and minimal distortion of cellular functions. Secondly, the probes must be stable under physiological conditions. For this purpose, some modifications are made on the probes whereby they are resistant to nuclease activity [157]. Thirdly, the probes must be offered a high signal-to-background ratio for molecular imaging. The differences between the signal intensity of the probe in ON and OFF states must be significant. Fourthly, the penetration of the probes into cells must be controllable. The delivery of a probe to specific sites of the cell depends on the probe length so that a long probe has a low penetration rate to the cell [158]. The unique properties of fluorescently labeled probes make them an effective choice in live-cell imaging. Molecular beacons by their inherent properties, such as ease of design and fabrication and low cytotoxicity, are extensively used for live-cell imaging [159]. However, degradation by nucleases, hairpin–hairpin binding, and non-specific interactions with proteins and small molecules have caused the molecular beacons to be a non-specific signaling probe [160]. Recent attempts to design suitable probes, which meet all live-cell imaging criteria, have resulted in the development of nanostructure-based probes. Wue and co-workers employed an electrostatic DNA nano-assembly process that realizes HCR for amplification in living cells. They used AuNPs as a core structure in the same format of the nanoflares which has high efficiency for the cellular delivery of DNA probes [161]. In another work, Wu and co-workers designed and optimized a hairpin DNA cascade amplifier for the detection of mRNA molecules in living cells [162]. In this strategy, the amplification principle was performed similarly to the conventional CHA method in which the recycled mRNA targets serve as new initiators for further signal amplification. Cheglakove et al. designed a FRET signaling platform based on HCR strategy for intelligent biosensing of microRNAs in live cells [163]. The HCR products had a binary FRET signal for providing a more sensitive assay by decreasing the background signal. Recently, Li et al. reported an HCR amplification strategy based on the specific properties of graphene oxide for simultaneously imaging two miRNA targets in living cells [164].

Challenges

Sensitivity is a critical parameter for imaging techniques, especially when the concentrations of the target nucleic acids in a single cell must be analyzed. This parameter depends on different factors, including background signal, reaction kinetics, type of amplification, and target accessibility. For in situ applications, background signal can be usually reduced by optimizing the concentration of labeled probes and increasing the stringency of the washing steps; however, in living cells, efficient fluorescent-based probes like FRET signaling systems and binary probes [165] can be used to suppress the background signals. Although these strategies improve the LOD value, they are not enough for an efficient biosensing in single-cell analyses. For example, molecular crowders and cellular compartments may appear as barriers to effective penetration of biosensor reagents in specific sites. It causes the sensitivity of the method to be reduced by slowing the reaction kinetic. However, employing nanoflare-based methods has helped overcome some of these shortcomings. Nanoflares, as "lab-on-a-nanoparticle" platforms [166], increase the local concentration of reaction partners and improve the reaction kinetic in cellular environments. Despite the fact that nanoflares make up for some of the difficulties of reagent delivery and enzymatic degradation, they suffer from a nontrivial limitation arising from non-amplification properties with a t:s value equivalent to X:X. In this regard, Ellington's group designed a molecular machine based on the coupling of CHA strategy with nanoflare platform (Fig. 13) to improve the sensitivity of nanoflares [5]. Although this new strategy has been evaluated for in vitro conditions only, it significantly enhances the sensitivity of the original nanoflare and suits for use in vivo applications.

To date, several signal amplification strategies, with improved sensitivities, have been used for live-cell imaging purposes [110, 162, 163]. However, the sensitivity parameter has been defaced in some cases because of the low target accessibility. To address this issue, the prediction of protein-binding sites and specific secondary structures in RNAs by some computing tools can help in designing specific probes for bare and unstructured regions of
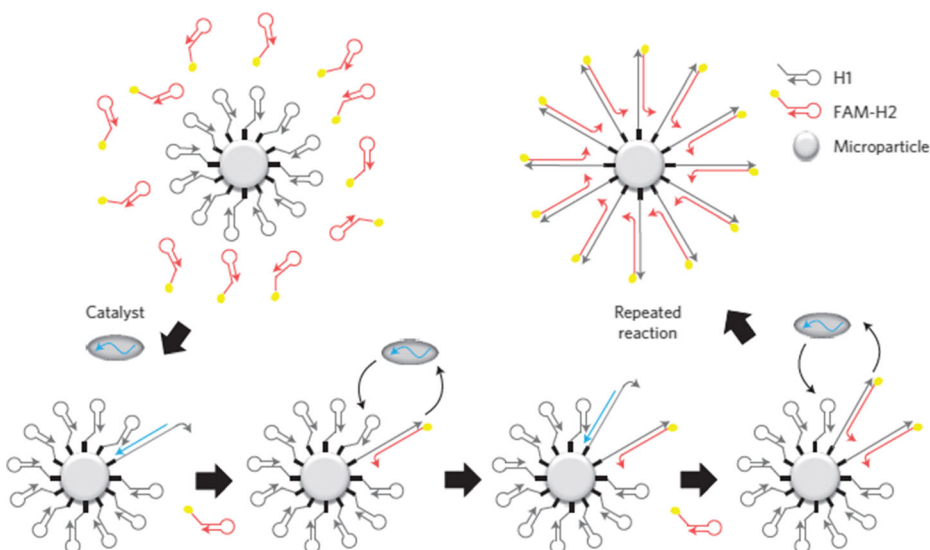

Fig. 13 Schematic representation of molecular machine based on the coupling of CHA strategy with nanoflare platform [5]

the target. As an alternative, applying different probes for start and terminal regions of the mRNAs may improve the chance of target accessibility. However, these strategies do not guarantee the accurate binding of the probes to specific sites on the target molecule. Recently, Ravan demonstrated a new strategy to unwind the folded RNAs by applying two helper oligonucleotides in ambient temperatures [19]. This strategy was performed for in vitro studies, but the efficiency of the method for in vivo imaging must still be evaluated.

Summary

Until now, various efficient and practical signal amplification methodologies have been designed to enhance the sensitivity of nucleic acid biosensing. In the present review, the main signal amplification strategies, including enzyme-assisted, DNAzyme-assisted, nanomaterial-assisted, and DNA nanotechnology-based methodologies have been surveyed. The capability of these platforms for nucleic acid analyses in different conditions, from in vitro to in vivo, was compared. A variety of these biosensing tools, particularly the ones that act based on DNA nanotechnology, are useful for application in inherently noisy conditions such as live cells. In addition, nanostructures like nanoflares, as signal enhancement tags and nanocarriers with the intrinsic properties such as great biocompatibility, high endocytosis efficiency, and enhanced affinities to the target analytes, have been increasingly used for the analysis of different RNAs in living cells.

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