

Nucleic Acids Analysis

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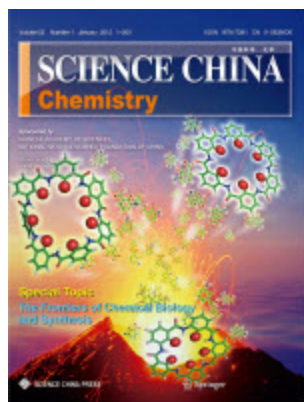
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Nucleic Acids Analysis

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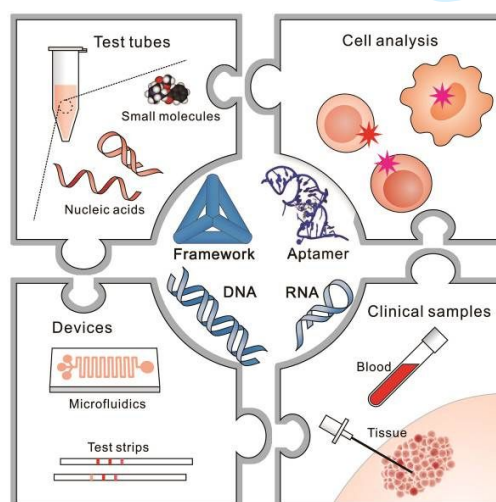
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Abstract

Nucleic acids are natural biopolymers of nucleotides that store, encode, transmit and express genetic information, which play central roles in diverse cellular events and diseases in living things. The analysis of nucleic acids and nucleic acids-based analysis have been widely applied in biological studies, clinical diagnosis, environmental analysis, food safety, forensic analysis. During the past decades, the field of nucleic acids analysis has been rapidly advancing with many technological breakthroughs. In this review, we focus on the methods developed for analyzing nucleic acids, nucleic acids-based analysis, device for nucleic acid analysis, and applications of nucleic acids analysis. We summarize representative strategies for the development of new nucleic acids analysis in this field, and discuss key advantages and possible limitations. Finally, we provide a brief perspective on existing challenges and further research development.

Keywords: nucleic acids analysis, signal amplification, DNA nanotechnology, aptamer, biosensing

Graphical abstract:



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1. Introduction

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are natural biopolymers of nucleotides that store, encode, transmit and express genetic information [1,2]. DNA is composed of a phosphate-deoxyribose sugar backbone and the nitrogenous bases adenine (A), cytosine (C), guanine (G), and thymine (T), while RNA has a ribose sugar backbone and another base uracil (U) instead of T. Single-stranded nucleic acids can form double helix structures when hybridizing to complementary sequences, following the Watson-Crick base-pairing rules (A:T or A:U, G:C) [3]. Both DNA sequences and RNA transcripts play crucial roles in diverse biological events, which can be exploited to serve as biomarkers for biological studies and clinical diagnosis. Electrophoresis and blotting are the most widely used analytical techniques for analyzing nucleic acids, which remain to be used to analyze the length or structure and sequence, respectively. Their sensitivities, however, are limited to the sub-microgram regime.

Inspired by intracellular DNA replication and RNA transcription, *in vitro* nucleic acid amplification techniques have been developed to improve signal response and detection sensitivity. They can be in general classified into two categories, thermal-cycle amplification such as best-known polymerase chain reaction (PCR) [4] and isothermal amplification [1,5,6]. The later one is especially useful for temperature-sensitive targets such as live cells and proteins. Bioanalysis at single-cell and even single-molecule levels have been achieved by employing these methods [7-9]. Moreover, high-throughput detection techniques, including microarray chip [10,11] and next-generation sequencing (NGS) [12,13], have been developed for simultaneous detection of thousands of and even more nucleic acid sequences of interest. The NGS technique can also be expanded for other biological and biomedical applications, including the analysis of non-nucleic acid targets [14] and the rapid screening of small-molecule drugs [15]. Several amplification methods have been utilized to improve the quality of sequencing library [16-18]. Furthermore, these nucleic acid techniques play important roles in cellular and *in vivo* applications. Live-cell imaging analysis with high signal gain has been realized by enzymatic or enzyme-free amplification methods [19-25]. Single-molecule visualization of nucleic acids or proteins in fixed cells or tissues has also been reported based on *in situ* DNA amplification methods [26-29].

DNA or RNA oligonucleotides have been selected as recognition probes such as aptamers and DNazymes by the systematic evolution of ligands by exponential enrichment (SELEX) [30,31]. Most of these probes bind to target molecules (proteins, small molecules, ions, etc.) with equal or even higher affinity and specificity than those of antibodies. Innovative bioanalysis methods based on aptamers or DNazymes have been proposed to enhance detection performance [32,33]. Unnatural nucleic acids, such as locked nucleic acids (LNAs) and peptide nucleic acids (PNAs), have been synthesized to improve the hybridization or binding stability and nuclease resistance [34,35]. Nucleic acids also work as basic elements in nanotechnology ascribed to their programmable and scalable feature [2,36,37]. They are used to construct various nanostructures and dynamic devices with diverse technological applications such as nanofabrication, biosensing and nanomedicine. DNA nanostructures have now been widely used to improve analysis performance. Furthermore, above-mentioned nucleic acid techniques are often integrated with portable or miniaturized devices including thermocyclers, microfluidic chips, test strips, and capillary platforms [38,39]. Commercial devices and assay kits have been available for practical testing. The applications of nucleic acids analysis have been expanded to clinical translation, environmental analysis, food safety and forensic analysis.

In the past two decades, explosive increase of research interest in this area has been witnessed along with massive publications. In this review, we summarize recent advances in nucleic acids and nucleic acids-based analysis, as well as device development and applications of nucleic acids analysis, and discuss the challenges and future prospects in this booming field.

2. Analyzing nucleic acids

2.1 *In-vitro* analysis of nucleic acids

2.1.1 Target amplification-based approaches

With the development of biotechnology and analytical technology, highly sensitive and selective approaches to amplify nucleic acids signals have been proposed to overcome the inherent defects of nucleic acids detection, such as low sensitivity, low selectivity and ultralow concentration. These methods include thermal cycling and isothermal amplification techniques. PCR is the most widely used thermal cycling technique for DNA amplification. The isothermal process includes enzymatic isothermal amplification reactions and enzyme-free DNA circuit signal amplifications. Enzymatic isothermal amplification reactions include strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA) and biological signal amplifications. Enzyme-free DNA circuit signal amplifications include hybridization chain reaction (HCR) and catalyzed hairpin assembly (CHA) (Figure 1).

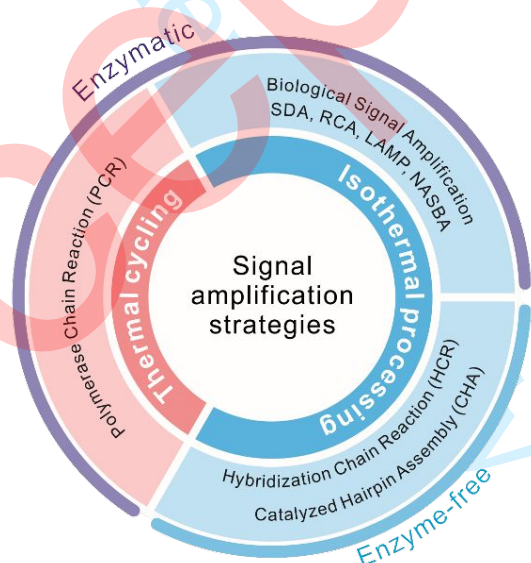


Figure 1. Signal amplification strategies for nucleic acids detection, including thermal cycling and isothermal methods. The isothermal methods mainly include enzymatic isothermal amplification reactions and enzyme-free DNA circuit signal amplification reactions.

PCR is the first developed nucleic acid amplification approach, which has been regarded as the “gold standard” to amplify and detect specific nucleic acid sequences through thermal cycling [40,41]. PCR technique was first conceptualized by Kary Mullis in 1985 [42], and has been rapidly developed as one of the most commonly used amplification approaches with high sensitivity and rapidity for *in-vitro* analysis of nucleic acids [43,44]. While the early generations of PCR-based detection technology are time-consuming and cannot be used to quantitatively detect target nucleic acids, especially RNA samples, quantitative reverse transcription PCR

(qRT-PCR) has been engineered for sensitive amplification and detection of target RNAs via dynamically fluorescence monitoring [45-48]. qRT-PCR allows the reverse transcription of target RNA to its complementary DNA, which realizes the effective amplification and quantitative analysis of the target RNA [49]. In addition, to further develop more sensitive and accurate PCR technology, digital PCR (dPCR) was emerged to actualize the absolute quantification for nucleic acid detection and nucleotide copy number variant analysis [50]. With dPCR technique, the absolute quantitative results can be calculated with higher sensitivity through the statistical analysis of the target nucleic acid signals [51]. By combining with microfluidic chips [52] and droplet-based technology [53], many commercial dPCR systems have been widely applied and significantly broadened the applications of dPCR.

PCR remains to be the most widely used technique for DNA amplification, which involves template strands to be amplified, repeated cycles of heat denaturation, hybridization with annealed primers to form duplex, extension by DNA polymerase with the aid of dNTPs [54-56]. Hence, PCR requires precisely controlled temperature cycling, complex and time-consuming sample preparation, which limits its applications in resource-limited conditions. To circumvent this barricade, isothermal amplification techniques were proposed. To detect target oligonucleotides, enzymatic isothermal amplification reactions have been come up. SDA is based on polymerization and scission, adapting to several sensitive DNA detection [57,58]. RCA refers to a circular oligonucleotide as a template. DNA polymerase replicates the template hundreds to thousands times to obtain a long single-stranded DNA product [59-61]. Another technique, LAMP, which is based on 4 primers being used to recognize the 6 regions on the template DNA to amplify nucleic acids at the range of 65 °C, can amplify the amount of DNA to 10^9 - 10^{10} in less than one hour [62,63]. In NASBA, three enzymes are used to detect RNAs at constant temperature 41 °C [64,65]. These enzymatic isothermal amplification approaches proceed at constant temperature, which spend less time than the thermal cycling approaches. Although these methods can reach high selectivity and sensitivity detection of oligonucleotides, they cannot adapt to amplify proteins, enzymes and small molecules *in vitro*. To detect these targets at low level, biological signal amplification technology has been put forward. Biological signal amplification techniques do not amplify targets *in vitro*, but improve the signal intensity of biomarkers or the amount of bioprobes to improve the sensitivity of detection. Biological signal amplification techniques include reactions based on nuclease nicking enzyme signal amplification [66-69] and DNAzyme signal amplification [70,71]. These methods are widely used in detection of metal ions, proteins and small molecules, and are dependent on enzymes suffering from high price and strict reaction conditions, e.g. certain temperature and buffer. To overcome this bottle-neck, enzyme-free DNA circuit signal amplification techniques have been proposed. HCR and CHA are the most widely used methods. HCR is the first reported enzyme-free DNA circuit signal amplified technique [72]. In HCR reaction, two DNA hairpins are stable in the solution until a target initiates the cascade of hybridization events to produce a linear, long double-stranded DNA (dsDNA) copolymer [73-77]. In CHA system, two stable DNA hairpins coexist in solution until a target is introduced. In the presence of the target, one of the hairpins hybridizes with target by toehold-mediated strand displacement, exposing the sticky end of hairpin 1 (H1), then hybridizing with the sticky end of hairpin 2 (H2) to form target-H1-H2 duplex. The duplex is unstable and the target is released to solution by H2 spontaneous disassembly to form H1-H2 complex. The released target initiates the next cycle to form the additional H1-H2 complex, then the target is used to amplify the signal

[78-81]. The most attractive feature of enzyme-free DNA circuit signal amplification techniques is that they are kinetic controlled reactions without requirement of enzymes.

2.1.2 Hybridization-based approaches

Hybridization-based detection platforms with high selectivity and excellent sensitivity have been developed based on Watson-Crick base-pairing rules. The most classic hybridization-based *in vitro* analysis is implemented by the hybridization of two complementary DNA strands (Figure 2(a)). By labeling functional groups or introducing nanomaterials on DNA strands, numerous strategies have implemented the analysis of nucleic acids [82-85]. The nucleic acid analogs (NAA), which are synthesized by modifying inter-nucleoside linkages or interspersing natural nucleobases with artificial nucleobases, also play important role in the hybridization-based *in vitro* analysis for their high selectivity and chemical stability (Figure 2(b)) [86-88]. In order to increase the selectivity, molecular beacon (MB), a single-stranded, stem-loop folded oligonucleotide chain structure with a donor (fluorophore) and an acceptor (quencher) conjugated to the opposite ends, is developed to detect target nucleic acids based on the switch of the stem-loop structure (Figure 2(c)) [89-93]. The MB is normally in the “fluorescence off” or closed state in the absence of target DNA. And it switches to the “fluorescence on” or open state after hybridizing with target DNA. In the system of duplex probes, the strand displacement reaction (SDR) can be used for *in vitro* analysis with high sensitivity, high controllability, and low interference (Figure 2(d)). Such an approach has been demonstrated to implement multiplexed detection for several classes of biomolecules [94,95].

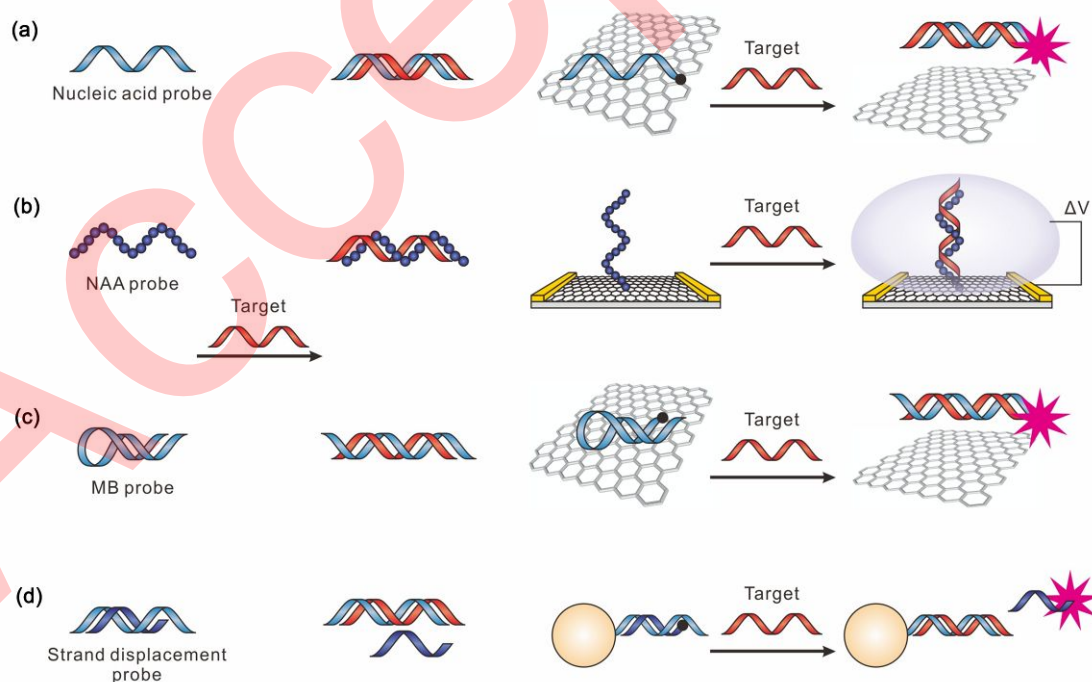


Figure 2. Representative DNA detection strategies based on nucleic acids hybridization. (a) The classic hybridization-based *in vitro* analysis strategy [83-85], Copyright 2013 Wiley-VCH. (b) The NAA analysis approach [86-88], Copyright 2014 American Chemical Society. (c) MB detection system [89-93], Copyright 2010 Royal Society of Chemistry. (d) The SDR detection strategy [94,95], Copyright 2014 National Academy of Sciences of the United States of America.

The sandwich assay has been widely applied for the detection of nucleic acids, since the Hassel *et al.* applied “sandwich hybridization” to investigate viral RNA transcripts in 1970s [96]. Typically, it often involves a target of nucleic acids inserting within two recognition probes [6]. To complete this assay, a recognition probe is modified onto surface to capture target nucleic acids from the solution via hybridization, while a second probe, coupled with signal reporter, such as radioactive isotopes, enzymes, fluorescein, and redox tags, is hybridized to a second region of the target nucleic acids. Upon the target nucleic acids binding to the recognition probe, the signal probe produces a readily sensitive measurable signal, enabling detection of the presence and concentration of target nucleic acids. Owing to simultaneous binding of the recognition probe to the target analyte, this method exhibits extremely high specificity [97]. More importantly, both surface bound probe and signal probe do not interact with each other in the absence of target, resulting in reduced noise and thereby ultimate low detection limits [98, 99].

Recent advances in the field of DNA nanotechnology have opened new possibilities to develop novel sandwich assays with impressive detection performance (Figure 3) [100-102]. For example, Lin *et al.* reported an E-DNA based sandwich assay by folding the tetrahedral framework-modified capture probe into stem-loop structure to further reduce the background signal and increase its detection sensitivity [103]. In the presence of nucleic acids target, the capture probe opens its stem-loop structure, generating a new sticky-end to hybridize signal probe, enabling this sensor detecting microRNA (miRNA) with high specificity in the diverse miRNA family members. Furthermore, Lu *et al.* introduced this E-DNA based sandwich assay into electrochemiluminescence resonance energy transfer (ECL-RET) detection system with high sensitivity and specificity by using $\text{Ru}(\text{bpy})_3^{2+}$ -modified silica nanoparticles (RuSi NPs) and hollow Au nanocages as ECL donor and acceptor, receptively [104]. In addition, Zuo *et al.* proposed a supersandwich assay by extending signal probe with a “sticky end” to further hybridize another target of nucleic acids [99,105]. Specifically, this hybridization of single signal probe between two target molecules results in formation of supersandwich structure with multiple labels, enabling impressive detection limit of this supersandwich assay.

Owing to the high stability, low cost, and convenient labeling, nanoparticles can also serve as signal probes for DNA detection to further improve the performance of sandwich assays [106-115]. For example, Zhang *et al.* reported a nanoparticle based “sandwich” detection strategy by immobilizing gold nanoparticles (AuNPs) with reporter probes that flank the target nucleic acids [116]. Specifically, hundreds of reporter DNA strands loaded on AuNPs enable a significant amplification by concentrating electrochemical signal of $[\text{Ru}(\text{NH}_3)_6]^{3+}$ proximal to the electrode surface, significantly enhancing its sensitivity and selectivity. Similarly, Liu *et al.* further applied this AuNPs based sandwich type strategy to detect exosomal miRNAs with neutrally charged PNAs instead of DNAs to further improve its sensitivity and specificity [100].

The development of framework nucleic acids (FNAs) provide a platform for patterning biosensing interfaces, resulting in uniform biorecognition layers to improve the hybridization efficiency in sandwich assay. As an elegant application, tetrahedral DNA frameworks (TDF) have been used as rigid scaffolds for orientated immobilizing DNA recognition probes. Fan *et al.* have shown that the DNA hybridization kinetics and thermodynamics were profoundly affected by tuning the size of TDF anchors. The hybridization rate and hybridization efficiency can be increased for 20-fold and 5-fold with TDF anchors, respectively, compared to the conventional single-stranded DNA anchors. When TDFs were used to develop sandwich assay, a detection limit

of 100 aM can be achieved, which excels most of the existing sandwich assays [117-119]. In addition, the intrinsic low cost of TDFs meets well the low-cost requirement of biosensor design because the TDFs can be assembled with only four DNA strands [120-122]. More importantly, the FNAs facilitated patterning of biosensing interfaces can be integrated into various sensing devices such as microfluidic devices and paper-fluidic devices [123,124], expanding its applications in many important fields such as miniaturized biosensors and integrated biosensors.

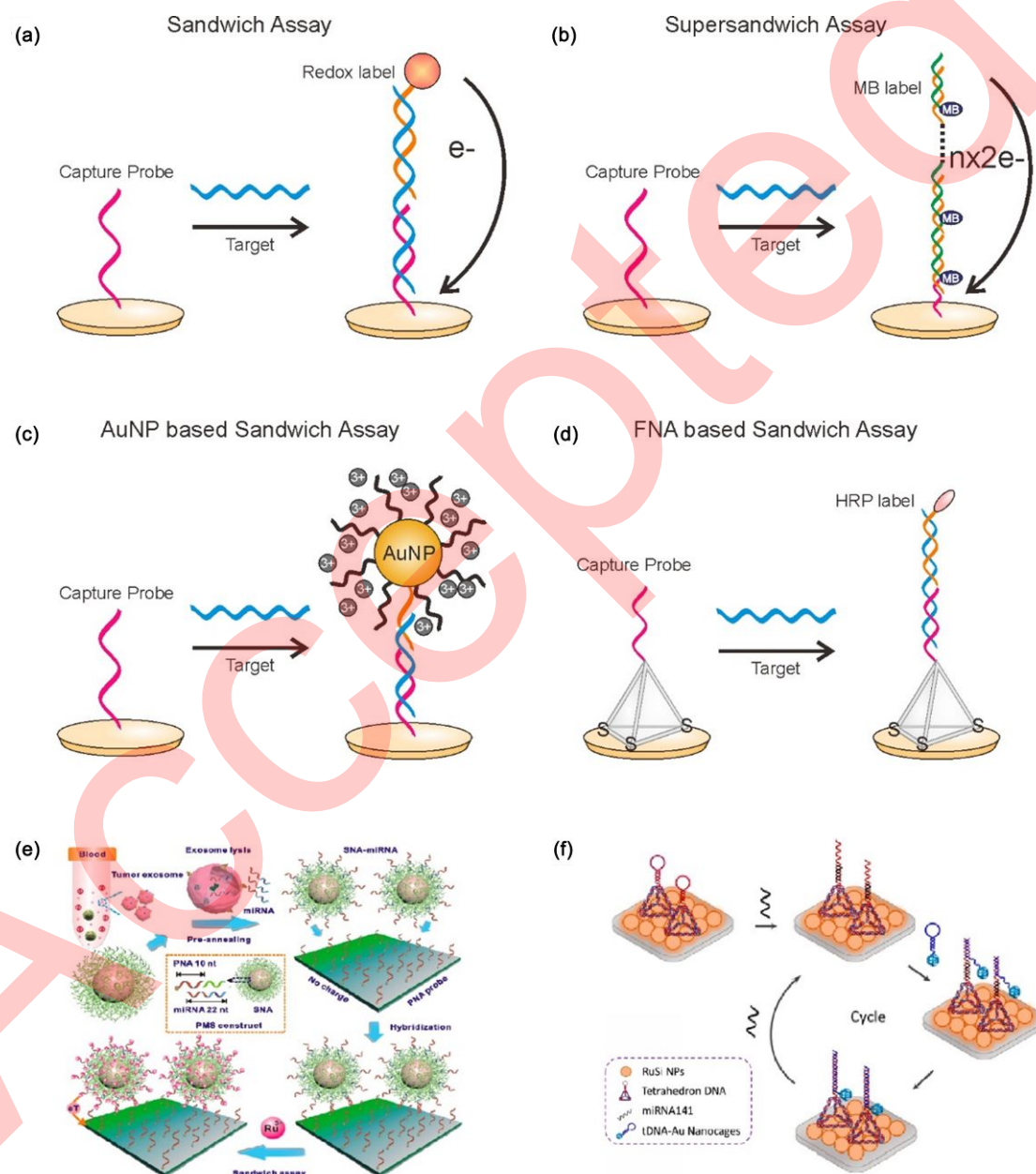


Figure 3. DNA detection based on the sandwich strategy. (a) Scheme of the traditional electrochemical nucleic acid sandwich assays. (b) Scheme of supersandwich assays for the nucleic acids detection. (c) Schematic of showing AuNPs amplified detection: DNA targets, detection probes loaded on AuNPs, and capture probes anchored at the electrode surface form “sandwich” complexes in the presence of target DNA. (d) Scheme of the FNA based E-DNA sensor with DNA tetrahedron. (e) Schematic illustration of miRNA detection using the PNA-miRNA-SNA (PMS) sandwich-based biosensor [95], Copyright 2019 American Chemical Society. (f) Schematic

demonstration of structure and modification process of the FNA based ECL Biosensor for miRNA detection [125], Copyright 2018 American Chemical Society.

2.1.3 Sequencing-based approaches

Sequencing is probably one of the most sophisticated applications of analyzing nucleic acids [126]. Many conventional analytical methods, such as electrophoresis separation [127,128], laser-induced fluorescence [127], fluorescence microscopy [128], electrochemical sensing [129], microfluidics [130], single-molecule detection [131], and the integration between some of these technologies, become the technological foundation of various types of DNA sequencers. DNA sequencing also highly relied on the advances of the understanding and engineering of enzymatic reactions, as well as the nucleic acid amplification techniques [132] to convert the sequence information into measurable optical or electrical signals [133,134]. The first drafts of the human genome were published nearly two decades ago [135], since then we have observed the explosion of genomic data along with the revolutionized instrumental development from the capillary electrophoresis-based Sanger sequencers [136] to high-throughput next-generation sequencers [137-140]. There have been many review articles that cover all aspects of DNA sequencing and its applications in studying genomic variations [141], transcriptomes [142], epigenomes [143], higher-order chromatin structures [144], protein-nucleic acid interactions [145], and diagnosis applications [146,147]. Here we emphasize three major trends that illustrate technological advances built upon analytical chemistry (Figure 4). First, long-read single molecule sequencing, such as the nanopore sequencing [148], has been rapidly developed and is catching the wave to provide solutions with unique advantages including the detection of viral DNAs or RNAs during outbreaks [149]. Although such approaches still face great challenges on the raw accuracy [150], and will not be able to replace prevalent NGS platforms in the near term, direct long-read detection scheme has been increasingly used as a synergetic tool to couple with short-read sequencing approaches for acquisition of long-range genomic information [151]. Second, large throughput sequencing will still hold the major role in sequencing business and need to be continuously evolved to reach higher throughput, lower cost, higher accuracy, and longer read length. New concepts of sequencing chemistry, such as error-correction code sequencing [13] and contiguity-preserving transposition sequencing [152,153], will greatly elevate the merits of high-throughput sequencing and benefit majority of applications with familiar experimental and analytical procedures. Third, the capability to sequence low-input samples allows us to explore nucleic acid information with higher spatial resolution. Single cell sequencing is becoming a routine process when dissecting a complex biosystem [154], and it has been highly depending on the advances of microfluidics [155,156], development of amplification methods [12,17,157], and the new functions of enzymes [158]. Meanwhile, spatial-resolved *in situ* sequencing will become a new frontier in biology and medicine, providing an integrative landscape of both spatial distribution and genomic variation at the single-cell level [159-161], or even finer scales [162], in the context of tissues or organs. The advent of new technologies in sequencing has shown great prospects for deciphering the secrets of life, and will continuously shed light on fundamental biology and medical applications.

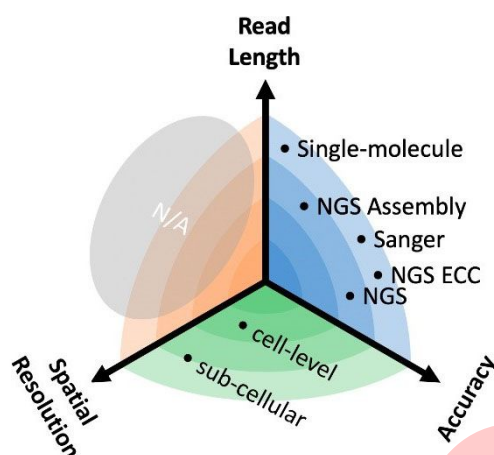


Figure 4. Sequencing technology developing trends. Most improvements have been observed in sequencing read length and accuracy (blue domain), while the progress in increasing sample spatial resolution is relatively slow (orange and green domains). No single technology excels in all three dimensions to date.

2.1.4 Mass spectrometry-based approaches

Mass spectrometry (MS) has long played a significant role in the characterization of constituents, modifications and sequences of nucleic acids due to its excellent performance in identification of compounds [163]. The fast advances of MS-based analytical methods make MS an increasing important tool in nucleic acids research. Specially, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) are the most widely employed platforms for analyzing nucleic acids.

Nucleic acids typically undergo hydrolysis, resulting in formation of small compounds, such as nucleotides, nucleosides, or nucleobases, followed by determination of these components with MS (Figure 5) [164]. The chromatographic retention time, mass-to-charge ratios and fragmentation MS spectra could afford the identification information of components from nucleic acids. Stable isotope internal standards were commonly used in MS analysis to improve quantification accuracy. Apart from the canonical nucleic acids, a number of modifications that play critical roles in regulating the structures and functions of nucleic acids have been identified in both DNA and RNA [165]. Analysis of these modifications shows great promise in clinical diagnosis and prognostics of certain diseases [166,167]. However, nucleic acid modifications generally exist in low abundance, and thus cause challenges in the MS-based analysis. In this respect, chemical labeling in combination with MS analysis has been proposed and proved to be an attractive strategy to increase the detection sensitivities of analytes (Figure 5) [168,169]. Along this line, a variety of chemical labeling-MS-based methods have been developed for the sensitive and accurate analysis of nucleic acid modifications with the detection sensitivities of nucleosides being down to subfemtomole or even attomole levels [170-175].

MS also plays an ever-widening role in determining sequence of oligodeoxynucleotides. The large nucleic acids generally have to be cleaved to short fragments for subsequent MS analysis (Figure 5). As for RNA, the nucleases of RNase T1 and RNase A are typically utilized to digest RNA to produce small oligonucleotide fragments followed by MS analysis [176]. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) normally create

protonated or deprotonated molecular ions in the form of $(M + nH)^{n+}$ and $(M - nH)^{n+}$, respectively [177]. To obtain the sequence information, the selected oligodeoxynucleotides need to be further fragmented. A typical fragment ion spectrum of an oligodeoxynucleotide is composed of a series of peaks. Starting from the 5'-terminus, a, b, c, and d fragment ions, are created by fragmentation at different positions of phosphodiester group in oligodeoxynucleotide (Figure 5) [163]. Meanwhile, z, y, x, and w fragment ions are produced from the 3'-terminus (Figure 5) [163]. The resulting series of 5'-terminal and 3'-terminal ions have characteristic mass differences, which are thereby employed to deduce the sequence of oligodeoxynucleotide [178]. In addition to the sequence information, the sites of modifications in oligodeoxynucleotides also can be obtained by MS/MS analysis, which is very valuable for the detection and mapping of DNA adducts in oligodeoxynucleotides [179]. Moreover, the capability of MS for sequencing oligonucleotides has also enabled the discovery and identification of various modifications in different RNA species [180]. With the development of advanced MS instruments, the mass resolution and detection sensitivity have been greatly improved. We envision that MS will play increasing important roles in analyzing nucleic acids.

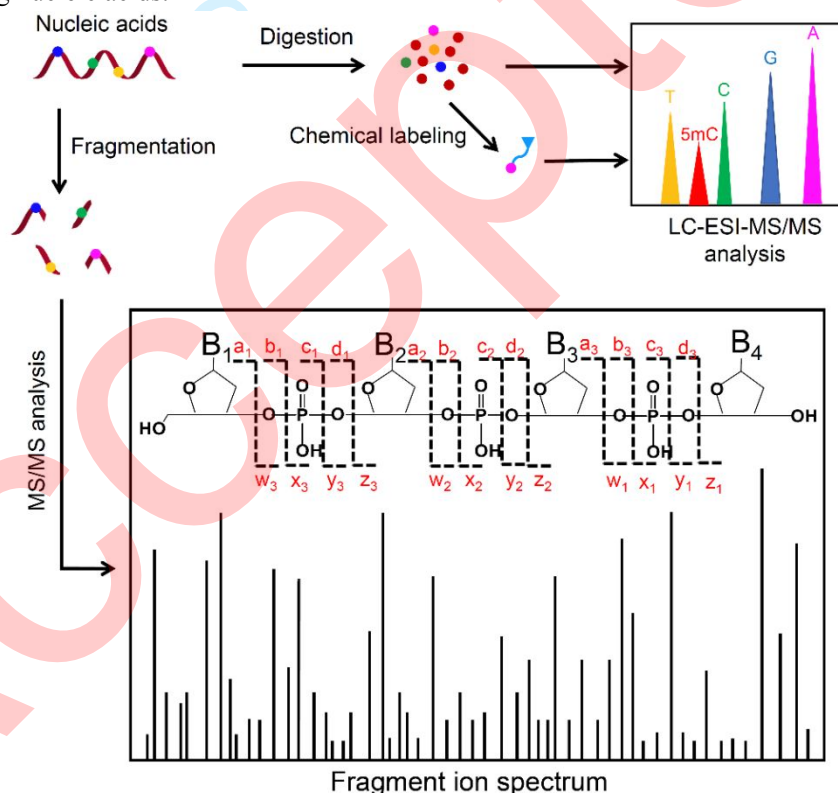


Figure 5. Schematic illustration of MS-based analysis of nucleic acids. Nucleic acids undergo hydrolysis to form nucleotides, nucleosides, or nucleobases followed by determination of these compounds by LC-ESI-MS/MS. Chemical labeling in combination with MS analysis can increase the detection sensitivities of these compounds. Nucleic acids also can be cleaved into short fragments and the sequence information of these oligodeoxynucleotides can be obtained by MS/MS analysis.

2.2 Intracellular and *in vivo* analysis of nucleic acids

2.2.1 Fluorescence

Fluorescence analysis has been widely used for the detection of nucleic acids of interest as a

non-invasive analytical method with high sensitivity and spatiotemporal resolution, which would be beneficial to understand the progress of physiology and pathology [181-184]. Using fluorescent probes, monitoring of nucleic acid molecules could be realized at the cellular and *in vivo* level on a real time scale. The fluorescent probes usually employ external fluorochromes with unique properties such as highly separated excitation/emission wavelengths, high quantum yield, long fluorescence lifetime, high extinction coefficient, and fluorescence anisotropy [185-188]. Furthermore, simultaneous detection of multiple targets can be achieved in the same sample by using different fluorochromes that can be spectrally resolved [189,190]. With these features, a variety of strategies have been designed for imaging and detecting nucleic acids with superb sensitivity, high selectivity and good biocompatibility in living cells and *in vivo* [190-194].

Chen *et al.* have developed a new activatable NIR fluorescence nanoprobe (QD-AI-GFLX) for efficient detection of dsDNA in aqueous solution and imaging of dsDNA in living cancer cells. As shown in Figure 6(a), only the HeLa cells incubated with QD-AI-GFLX produced strong intracellular NIR fluorescence upon interaction with dsDNA in the nucleus, indicating that QD-AI-GFLX was capable of fluorescence imaging of endogenous dsDNA in living cells [195]. Han and his coworkers reported an organelle-trappable DNA sensor (SG-RB) for dual-color imaging of mitochondrial DNA (mtDNA). As shown in Figure 6(b), SG-RB selectively bound mtDNA in mitochondria, thereby giving rise to turn-on green fluorescence [196]. Tang and his coworkers presented a multicolor nanoflares based novel nanoprobe that can detect and image three kinds of tumor-related mRNAs in living cells simultaneously. The fluorescent signals observed under confocal laser scanning microscopy (CLSM) were consistent with the levels of tumor-related mRNA gene expression, which revealed that this novel nanoprobe could be used to discriminate cancer cells from normal ones (Figure 6(c)) [197]. In addition to the intracellular detection of nucleic acids, the imaging and analysis of nucleic acids *in vivo* are also reported and help decipher biology. Jiang *et al.* developed a novel tripartite DNA probe (Y-H1-H2-FAP) that enabled fluorescent RNA imaging in living mice via an *in vivo* HCR circuit. The intense fluorescence signal observed for the HeLa tumor-bearing mice (Figure 6(d)) and the fluorescence intensities (Figure 6(c)) in the tumor regions of interest suggested the overexpression of microRNA-21 (miR-21) in tumors [198]. Li and his coworkers developed a DNA nanodevice (PBc-UN), permitting NIR light triggered, spatiotemporally controlled miRNA imaging in living cells and animals. Stronger fluorescence was shown by the PBc-UN + NIR treated group at the tumor site than the PBc-UN treated group (Figure 6(f) and 6(g)), achieving remotely activated miRNA imaging in tumors [199].

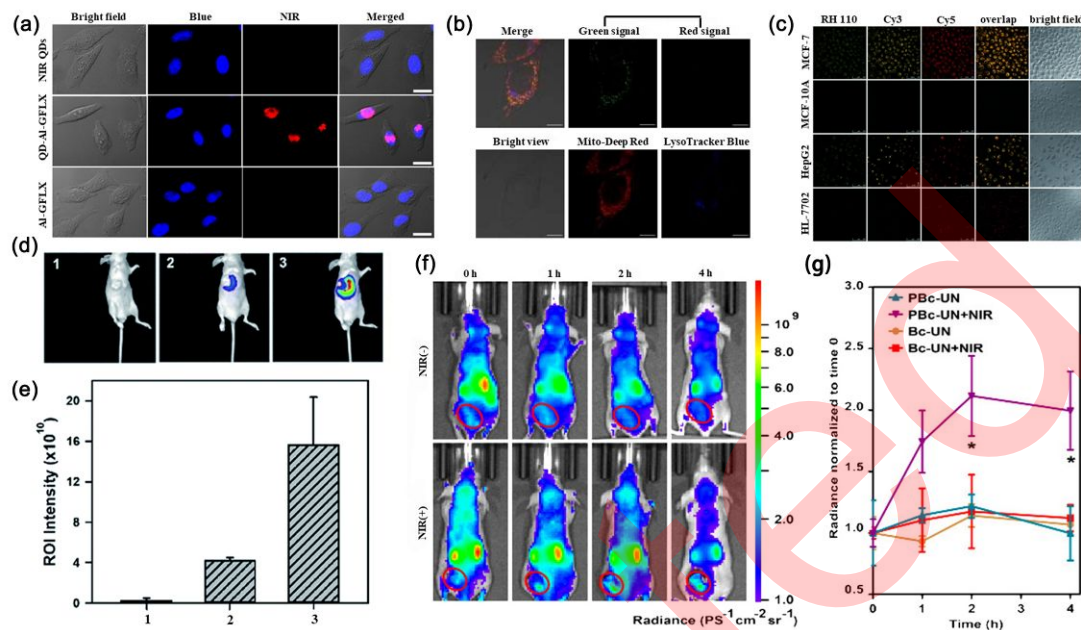


Figure 6. Intracellular and *in vivo* fluorescence analysis of nucleic acids: (a) Fluorescence imaging of dsDNA in living HeLa cells [195]. (b) “Turn-on” fluorescence imaging of mtDNA with SG-RB [196]. (c) Intracellular imaging of c-myc mRNA, TK1 mRNA, and GalNAc-T mRNA under CLSM [197]. (d) *In vivo* fluorescence images of HeLa tumor-bearing mice with injection of physiological saline injection (1) and Y-H1-H2-FAP probe (3) and MCF-7 tumor-bearing mice with injection of the Y-H1-H2-FAP probe (2) [198]. (e) Mean fluorescence intensities of the treated mice in (d). (f) Whole-body fluorescence imaging of HeLa tumor-bearing mice after intravenous administration of PBc-UN with or without subsequent NIR illumination. Red circles indicate tumor sites. (g) Quantification of the fluorescence intensity at the tumor sites in (f) [199].

2.2.2 SERS

Raman spectroscopy has attracted wide attention due to its prominent chemical specificity that provides fingerprint information of various molecules [200]. However, the inherently weak scattering characteristic limited its biological application. Fleischmann *et al.* firstly reported the surface enhanced Raman scattering (SERS) phenomenon at roughened silver substrates. Up to date, SERS has been considered as potential tools for analytical sciences and biomedical applications because of its remarkable sensitivity of detection and undisturbed property to surrounding interference factors [201]. SERS sensing mainly based on two strategies, direct detection and indirect detection with Raman tags. Direct detection of analytes is performed by monitoring the characteristic fingerprint information of the analytes' immediate vicinity by the enhanced substrates (such as noble metal nanoparticles). The indirect determination of analytes in biological samples depends on Raman tags that could specifically respond to or combine with the analytes, which is suitable for sensing analytes in complex samples [202].

Since Kneipp *et al.* firstly reported SERS applied in the direct detection of biochemical components in a single cell in 2002, SERS has been blossomed into intracellular analysis and *in vivo* imaging [203]. SERS can provide a wealth of components and structural information, as well as structural changes like mutations in DNA strands of the biological samples. Therefore, various studies about label-free direct detection of nucleic acids were reported [204,205]. For example,

Kneipp *et al.* monitored the cellular SERS signatures including nucleotides at different time points after the uptake of AuNPs [206]. Gold or silver nanoparticles also can serve as specific SERS tags based on the surface-enhanced Raman signature of an attached reporter molecule to highlight cellular structures and indirect detection of the characteristic analytes [207,208]. MiRNAs are a non-coding small RNAs that are considered as diagnostic markers or therapeutic targets of diseases [209]. Various nanostructure-based SERS tags were developed to improve the detection signals for sensitively and precisely determining miRNAs [206,209-212]. For example, Kuang *et al.* developed real-time *in situ* determination of the miRNA in living cells using self-assembly nanorod dimers. Distinct intense SERS signals were introduced by the self-assembly of plasmonic dimers simultaneously caused by miRNA [212]. GO-Au nanostructures were coupled with hybrid DNA linkers to detect miR-21 and surface glycoprotein in living cells with great selectivity and stability, in which the SERS signal would turn off as the concentration of intracellular miR-21 increased [210]. This research group also reported SERS and up-conversion fluorescence imaging platform for quantitative detection of the miR-21 and telomerase in living cells based on DNA-programmed AuNP dimer-UCNP core-satellite assemblies, in which the complementary DNA fragments for miR-21 were localized in the gaps of Raman tag-labeled AuNP dimers, while the hybridization of the complementary DNA fragment to miR-21 caused the AuNPs to separate, resulting in SERS signal turn off [210]. Moreover, based on the turn-on strategy for miR-21 detection, Liu *et al.* reported a series of methods, such as target triggered self-assembly method and target-mediated nanoparticle dimerization strategy. In detail, the authors designed dimeric AgNPs for quantitatively monitoring miRNAs in single cells, which can be controllably assembled to large aggregates for the formation of hot-spots, thus significantly enhancing the brightness of the Raman tags and achieving accurate detection of miRNAs at single-biomolecule level [213]. They also developed a target-mediated plasmonic dimerization method for *in situ* imaging of miRNAs in living cells based on the background-free Raman tags (alkyne, C≡C; nitrile, C≡N), with single peaks in the Raman-silent region and no overlap within the cellular fingerprint region. With the strategy, the electromagnetic hot spots would be built responsively to the target miRNAs, leading to significant enhancement of Raman scattering, thus reducing the interference of background signals [211].

Up to now, SERS technology has been widely applied for *in vitro* and cellular nucleic acid analysis [204]. However, *in vivo* nucleic acid analysis based on SERS methods is still extremely challenging. *In vivo* nucleic acid analysis is crucial for monitoring health, predicting the occurrence and progression of disease, and revealing potential targets for treatment. Tuan Vo-Dinh *et al.* reported *in vivo* nucleic acid detection by implanted a SERS sensor in the skin of pig based on a SERS signal turn-on strategy. The turn-on sensing system consisted of a plasmonic nanostar modified with a Raman tag-labeled DNA fragment and a complementary DNA fragment. The SERS signal was kept on turn-off status as the Raman label departed away from the nanostar surface, and would be enhanced significantly (turn-on status) when exposed to the target nucleic acids. The authors also demonstrated *ex vivo* SERS detection of target nucleic acid by implanting the SERS sensors in the human skin grafts [214], showing great potential for clinical translation applications of SERS technology.

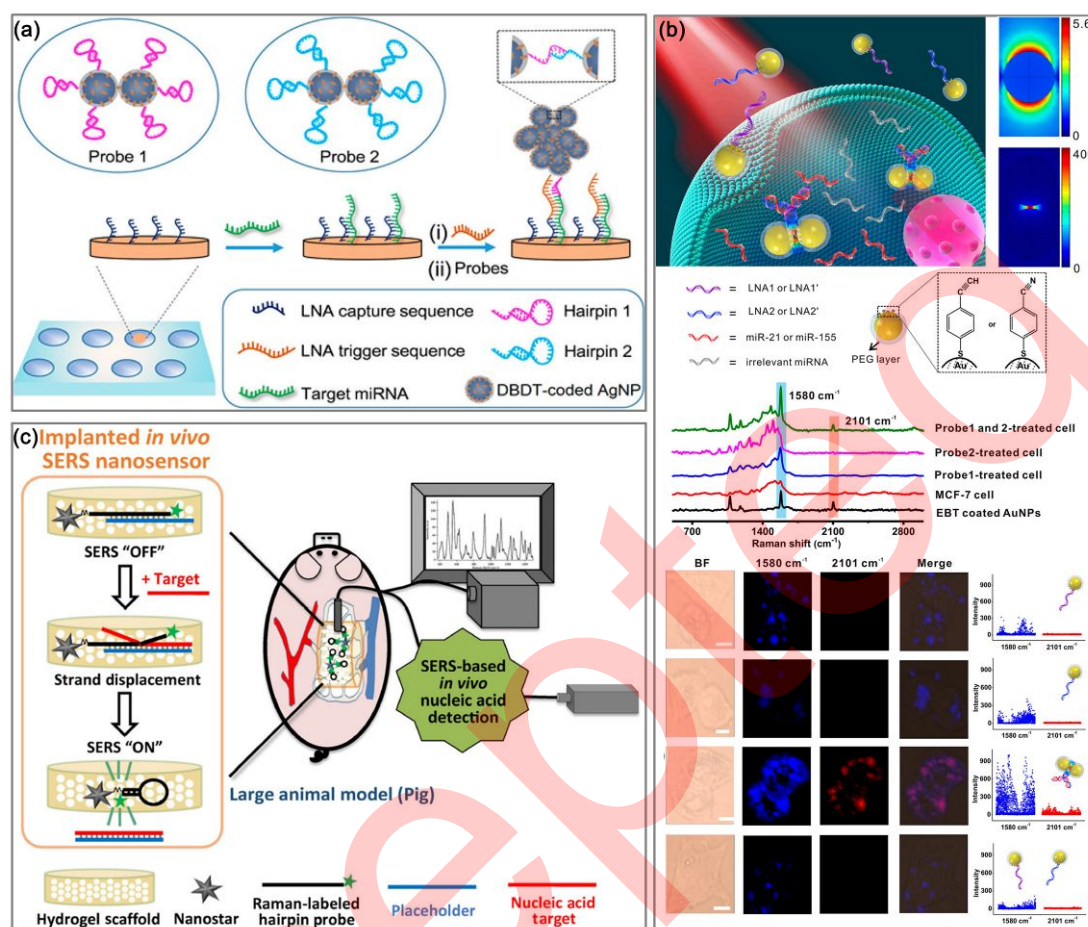


Figure 7. SERS strategies for nucleic acid detection. (a) Quantitatively miRNA detection using dimeric AgNPs, which can be assembled to large aggregates upon the presence of miRNA. Adapted with permission from ref [214], Copyright 2017 American Chemical Society. (b) In situ imaging of miRNAs in living cells, based on hot spots building in the presence of miRNAs. Adapted with permission from ref [211], Copyright 2017 American Chemical Society. (c) *In vivo* nucleic acid detection by implanted a SERS sensor in the skin of pig based on a SERS signal turn-on strategy. Adapted with permission from ref [214], Copyright 2018 Springer-Verlag.

2.2.3 Electrochemistry

Electrochemical approaches can offer rapid, low-cost and simple platform for the analysis of intracellular nucleic acids. Up to date, many electrochemical techniques have been developed for the intracellular sensing of nucleic acids in the diagnosis of human cancers [215-218], clinical and pathological diagnosis of genetic or infectious diseases [219-225], environmental monitoring, food analysis [226-230], and so on. For example, an amplification-free electrochemical approach was proposed for the analysis of FAM134B messenger RNA in tissues samples from patients with oesophageal carcinomas using a screen-printed gold electrode [217]. Such approach avoided the tedious fabrication procedure of electrode and the analytical performance accorded with a standard qRT-PCR analysis. Quantitative data is normally necessary for genetically modified organisms in foods to evaluate whether foods in trade are in compliance with particular specifications legally established. Monitoring of genetically modified organisms in food was achieved by electrochemical detection of magnetically-entrapped DNA sequences by multiplexed enzymatic

labelling from complex samples [226]. Generally, these methods need to lyse the cell to extract the intracellular nucleic acids, which may prevent the knowledge of prior or future states of the cells [231]. It is still critical to observe the intracellular nucleic acids within living cells. Except fluorescence imaging, electrochemical technique has also been employed for intracellular sensing *in situ* owing to the excellent analytical features. For example, a switchable electrochemical sensor was designed for survivin mRNA in living cells on the basis of the changes of electron transfer efficiency between the electrode surface after reaction with the mRNAs and ferrocene at the end of DNAs [232].

In addition to the abovementioned potentiometric and amperometric assays, ECL technique that combines the advantages of electrochemical and chemiluminescence methods is also increasingly being used for intracellular sensing of nucleic acids because of the advantages of high sensitivity, low background, and good temporal and spatial resolution [233]. For example, an ultrasensitive wireless ECL assay was proposed for the detection of c-Myc mRNA in breast cancer cells on an indium tin oxide bipolar electrode in a poly (dimethylsiloxane) microchannel [234]. Such ECL biosensor could accurately quantify c-Myc mRNA copy numbers in living cells. ECL imaging of intracellular miR-21 in single HeLa cells was achieved by EMCCD using H₂O₂ as co-reactant and luminol as ECL active material [235]. To the best of our knowledge, it is the first reported example to employ the high resolution ECL-microscopy for imaging of intracellular miR-21.

3. Nucleic acids-based analysis

In addition to above-mentioned nucleic acids targets, nucleic acid elements also enable the bioanalysis of non-nucleic acid targets, including ions, metabolites, proteins, and even cells. This feature contributes to the nucleic acid-based molecular recognition elements such as aptamers and DNAzyme, which can bind to their targets with strong affinity and high specificity. Moreover, benefiting from their programmable and scalable features, nucleic acids can be constructed into DNA/RNA nanostructures, which can significantly improve the analysis performance of probes. In this section, these nucleic acid elements including aptamers, DNAzymes and DNA/RNA nanostructures are first introduced, then several strategies for their bioanalysis application are discussed, and finally some representative examples for the bioanalysis of various kinds of targets are provided.

3.1 Nucleic acid elements for molecular recognition

3.1.1 Aptamers

Nucleic acid aptamers are oligonucleotide sequences of DNA or RNA. The aptamer can achieve specific recognition due to its spatial structure that matches the target molecule [236]. Aptamers are usually obtained by SELEX screening [237]. Specifically, nucleic acid libraries were incubated with target molecules to obtain conjugates of aptamers and target molecules [238,239]. Then the unbound sequences were eluted. After multiple cycles of selection, aptamers with high affinity can be obtained [240].

In recent years, nucleic acid aptamers have been widely used in bioscience and biomedicine due to their high affinity, specific binding ability and excellent programmability [30,241]. At present, researchers have synthesized and screened a variety of nucleic acid aptamers that can specifically bind to biomarkers such as antigens [242], biological enzymes [243], cell surface receptors [244]

and nucleic acid molecules. For example, AS1411 is a star DNA aptamer that binds nucleolin with high affinity, thereby inhibiting cell proliferation and achieving the effect against cancer [245].

In addition to using nucleic acid aptamers for recognition analysis, some researchers have begun to introduce functional groups into nucleic acid libraries to obtain chemically modified aptamers [37]. Compared with traditional aptamers, chemically modified aptamers have a richer spatial conformation, which increases the possibility of obtaining suitable aptamers from nucleic acid libraries [37,246]. At the same time, the introduction of chemical modification is expected to expand the function and application of chemically modified aptamers [247]. An example is a chemically modified aptamer containing 2-amino-3-nitropyridin-6-one (denoted as Z) and imidazo[1,2-a]-1,3,5-triazin-4-(8H)-one (denoted as P) bases. Many researchers have introduced Z and P bases into nucleic acid libraries. The desired chemically modified aptamer is screened from the library (Figure 8) [248]. Currently, chemically modified aptamers have been widely used to recognize and analyze more biomarkers due to their high affinity and specificity.

Furthermore, chemically modified nucleic acids cannot be recognized by traditional nucleases due to the introduction of chemically modified nucleotides. As a result, the biological stability of the nucleic acid sequence is greatly improved [249]. There are also some chemically modified nucleotides that can be complementary paired with traditional natural nucleotides, which can further improve the stability of nucleic acid aptamers based on “locking” the spatial structure of the aptamers.

Numerous studies have shown that nucleic acid aptamers are widely used as multifunctional tools for recognition, analysis, drug delivery, cancer treatment and other fields [30,250]. Among them, the introduction of functional groups into nucleic acid aptamers is expected to further expand the structure and function of aptamers. These studies will provide new opportunities for future research and development of excellent analysis equipment and disease treatment drugs.

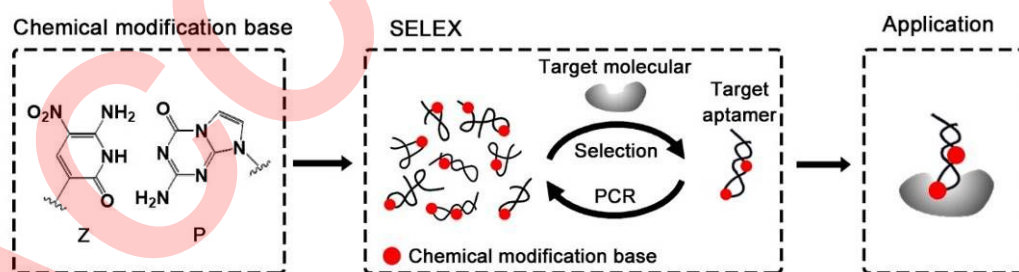


Figure 8. Generation and selection of DNA aptamer containing chemically modified bases towards recognition, analysis, drug delivery, cancer treatment application. An example is a chemically modified aptamer containing 2-amino-3-nitropyridin-6-one (denoted as Z) and imidazo[1,2-a]-1,3,5-triazin-4-(8H)-one (denoted as P) bases.

3.1.2 DNAzymes

With the discovery of ribozymes in the early 1980s, the notion that all enzymes are proteins was changed. Ribozymes can catalyze biochemical transformations in the absence of proteins. The use of ribozymes for sensing was less explored due to the poor stability and high costs of RNA. In 1994, the first catalytic DNA (DNAzyme) was isolated with the RNA cleavage activity (Figure 9(a)) [251], and its activity requires Pb^{2+} as a cofactor while other metal ions cannot catalyze the reaction [252]. The DNAzyme was obtained by *in vitro* selection from a large random library

containing around 10^{15} DNA sequences, and the process was quite similar to aptamer selection also including the isolation of active sequences followed by PCR amplification.

Many DNAzymes require specific metal ions [253]. Li and Lu took advantage of it and reported a DNAzyme-based biosensor for Pb^{2+} detection [254]. This sensor was composed of an RNA containing substrate strand bearing a fluorophore label hybridized to a quencher labeled enzyme strand (Figure 9(e)). In the hybridized state, the fluorescence was low. Upon cleavage of the substrate and release of the fluorophore bearing fragment in the presence of Pb^{2+} , a time-dependent fluorescence increase was obtained [255]. Such DNAzyme-based sensors are highly sensitive and selective, and they solved the problem of detecting metal ions, which is challenging for aptamers and antibodies. Later, *in vitro* selections were intentionally carried out in the presence of different metal ions such as UO_2^{2+} (Figure 9(b)) [256], Na^+ (Figure 9(c)) [257], and lanthanides (Figure 9(d)) [258], all achieved highly selective sensors for the target metal ions. By measuring catalytic activity, the problem of non-specific DNA binding by metal ions can be largely avoided to further increase specificity [259]. To further improve the chemical diversity of DNAzymes, chemically modified DNA was used such as phosphorothioate DNA [260], modified DNA bases [261], and synthetic metal ligands [262].

The same selection method has also been applied to non-metal targets. For example, an early DNAzyme reported by the Breaker group requires L-histidine for its activity (Figure 9(f)) [263]. Later, the Li group has isolated a series of DNAzymes that can detect specific bacterial strains by selecting against their crude extracellular matrix [264,265]. In addition to the DNAzymes for RNA cleavage, another type of commonly used DNAzyme has peroxidase-like activity [266]. These DNAzymes often have a G-quadruplex binding hemin as a cofactor (Figure 9(g)). They are attractive in terms of replacing horseradish peroxidase in immunoassays for signal amplification. Since DNAzymes are programmable in structure and can achieve site-selective labeling, they are more versatile than protein-based enzymes for sensor design.

DNAzymes with other types of activities have also been used for analytical applications. For example, a sequence named T30695 was recently discovered to catalyze porphyrin metalation in the presence of Pb^{2+} (Figure 9(h)). By inserting Zn^{2+} into the porphyrin substrate, a red-to-orange emission color change was observed [267]. DNAzymes for oxidative and hydrolytic cleavage of DNA were developed for measuring Cu^{2+} [268] and Zn^{2+} [269], respectively. DNAzymes with phosphatase and ligase like activities are also known and they can also be potentially used for signaling or target recognition in sensors [270,271].

Overall, DNAzymes can be used for both target recognition and for signal generation and amplification. Thus, they are very useful molecules for designing biosensors, especially for metal detection. DNAzymes are complementary to aptamers in terms of target recognition, and they can also be combined to obtain aptazymes to have the advantages of both [272].

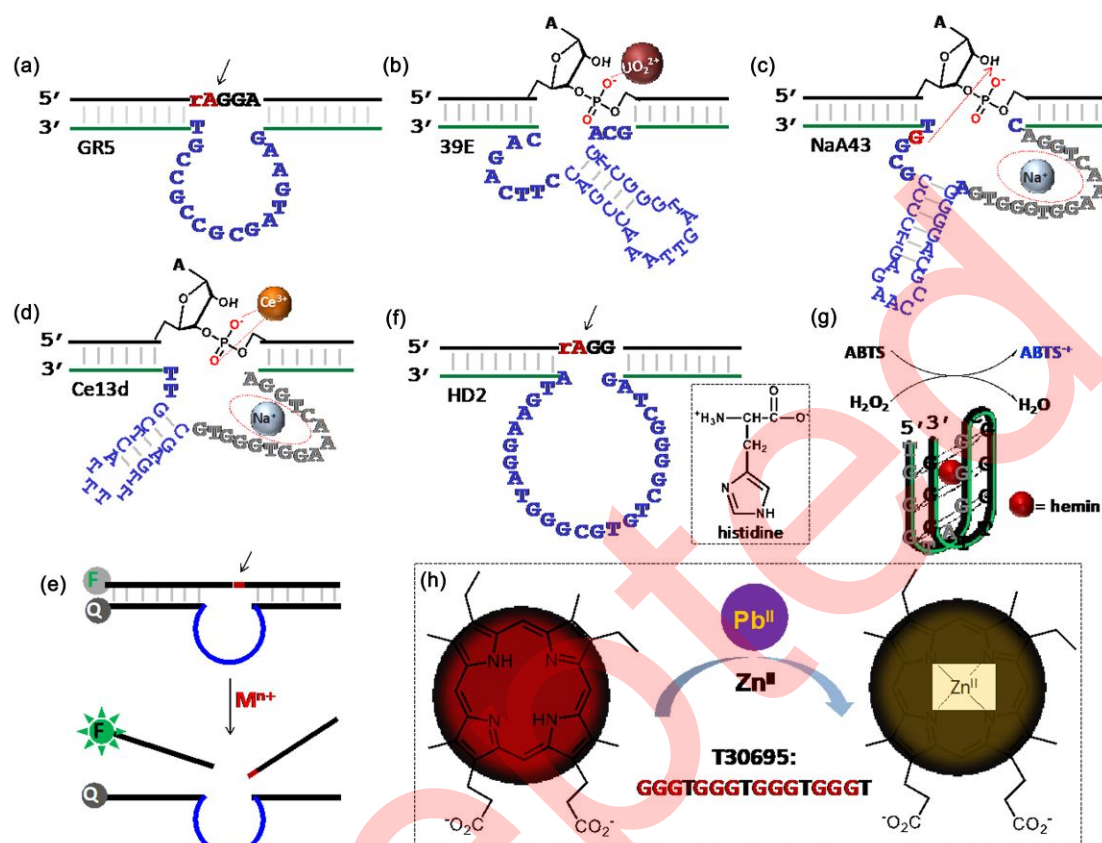


Figure 9. DNAzymes for molecular recognition. (a) The first reported RNA-cleaving DNAzyme named GR5 specific for Pb^{2+} . The secondary structures of representative DNAzymes requiring. (b) UO_2^{2+} (39E), (c) Na^+ (NaA43), (d) lanthanide ions (Ce^{3+}), or (f) L-histidine (HD2) as cofactors. (e) The cleavage-induced fluorescent beacon based on RNA-cleaving DNAzymes. (g) The G-quadruplex DNAzyme/hemin complex with peroxidase-like activity catalyzing the oxidation of ABTS in the presence of H_2O_2 into $ABTS^+$. (h) T30695 can catalyze porphyrin metalation in the presence of Pb^{2+} and induce a red-to-orange emission change upon inserting Zn^{2+} .

3.1.3 DNA/RNA nanostructures

The analysis aiming at targets with larger dimension or higher complexity (e.g. protein, biochemistry pathway and cell membrane) requests accordingly larger molecular objects to accommodate the geometric, procedural and affinity demands. DNA/RNA nanostructures have exhibited the corresponding properties and great potentials in the realm of biochemical analysis.

Initiated by Nadrian Seeman in the 1980's, the concept of DNA nanotechnology [2,273] inspired scientists to design nanoscale structures precisely assembled with programmable DNA oligonucleotides (Figure 10(a)). Different from aptamer or ribozyme whose functions are embedded in the sequence determined secondary structure of a single (sometimes more than one) DNA/RNA strand, the construction of DNA nanostructures requires less in sequence specificity, but more in geometrical rules (such as the distance between crossovers) to regulate the hybridized DNA segments to form proper spatial arrangements. Simple DNA nanostructures designed in the early days, commonly termed "tiles", are able to further assemble into larger structures (e.g. 1D tubes, 2D arrays and 3D crystals) with certain freedom in 1~3 directions to grow infinitely [274,275]. To fabricate more specific structures, DNA origami [248,276] and Single-Strand Tile

(SST) [277,278] techniques have made great progress in the last decade. They sequester hundreds of DNA oligos recognizing and binding to a single-stranded DNA scaffold or to each other to form the pre-designed structures with very high fidelity and efficiency. Various geometrical shapes across 1D to 3D (e.g. tubes, 2D patterns, bricks, spheres, webs etc.) have been realized with controllable parameters (e.g. distance, angle, curvature) in nanometer precision. Moreover, by employing dynamic DNA nanotechnology [279-281], properly designed DNA structures can transform between two or more isomers mostly through SDR that triggered by physical or chemical stimulus (e.g. photons, pH changes, ions, DNA oligos etc.). The above progresses in DNA nanotechnology suggest the unlimited possibility of engineering arbitrary nanodevices for specific tasks in both basic and applicable researches.

RNA molecules, in principle, can be manipulated to fold or assemble into various nanostructures just like DNA with a few distinct parameters [282]. However, considering the valuable cellular functions of RNA, which rely on their sequences and secondary structures, it makes less sense to assemble a static RNA structure using dozens of commercially synthesized RNA oligos with arbitrary sequences at high cost. Thus, the field of RNA nanotechnology [283-285] has been focusing on achieving higher order RNA structures through natural RNA motifs and their association methods, such as kissing loops and sticky ends (Figure 10(b)). Although the architecture species and constructions are not as impressive as the DNA nanostructure family, RNA nanostructures preserve the outstanding ability of cellular amplification and *in-vivo* assembly [286,287]. Publications in recent years indicate that the merging of DNA and RNA nanotechnology as a current trend may eventually integrate the structural programmability and the bio-functionality of the two kinds of nucleic acids [288-290].

The three major advantages of DNA/RNA nanotechnology (Figure 10(c)), including the abilities of multiple components arrangement, precise spatial positioning and dynamic structure regulation, can improve analysis performance in many ways. First of all, the readout fluorophores (or other molecules) can be easily introduced into DNA/RNA nanostructures with multiple species and copies, which significantly increase the number of detecting channels, and enhance the signal intensities [291,292]. Second, the DNA/RNA nanostructures possess numerous sites on surface with controllable distance to display multiple recognition motifs (e.g. aptamers, antibodies) that enhance the cooperative binding affinity for the target biological analytes [293,294]. Third, employing DNA/RNA nanostructures as frameworks, other nanomaterials (e.g. carbon nanotubes, metal nanoparticles, quantum dots etc.) and biomolecules (e.g. peptide, enzyme, antibody, lipid etc.) can be recruited and rearranged to establish novel analytical platforms with inherited properties (e.g. chirality, surface plasmonic resonance, catalytic activity etc.) [295]. Moreover, DNA/RNA nanostructures are capable of generating motions or investigating dynamic molecular behaviors [296,297]. Last but not least, recent studies in DNA nanostructure induced cell uptake and *in-situ* metabolism tracing suggest a huge potential of using DNA /RNA nanotechnology to facilitate *in vivo* investigation and nanomedicine discoveries [298-300]. In summary, DNA/RNA nanotechnology offers analytical chemists a convenient toolbox to choose or build up devices for better target detection, signal extraction and sample examination.

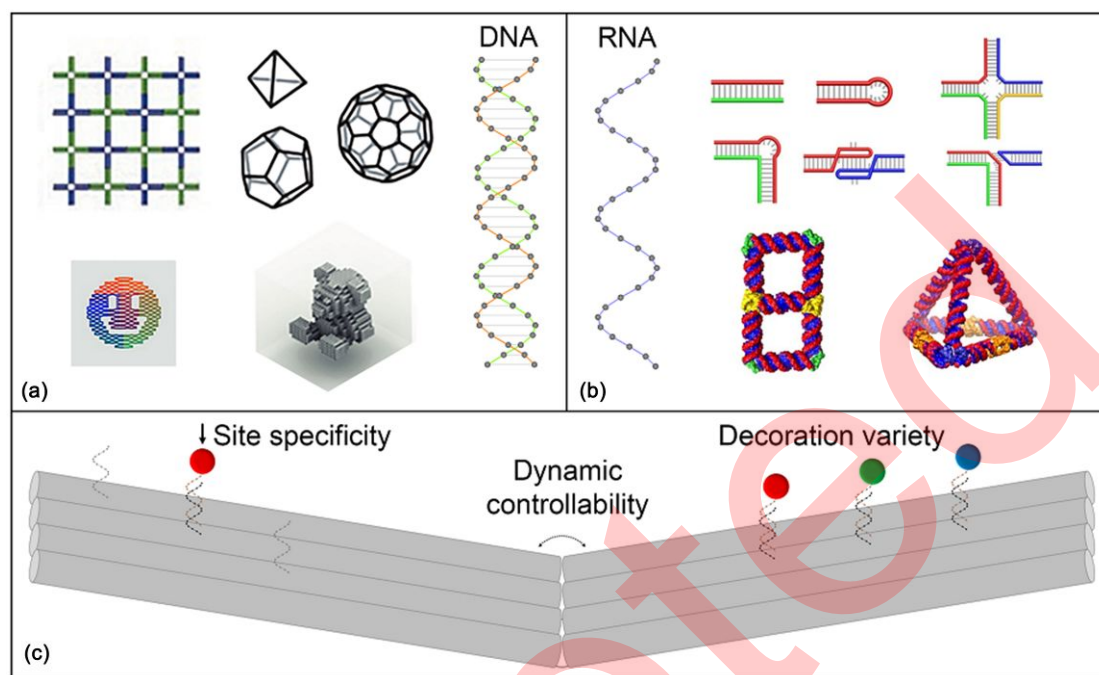


Figure 10. Schematic illustration of DNA/RNA nanostructures. (a) Nanostructures constructed by DNA in two and three dimension. (b) Motifs, binding/folding principles and 2D/3D nanostructures based on RNA molecules. (c) DNA/RNA nanostructures can be precisely functionalized and manipulated for sensing and many other purposes.

3.2 Strategies for bioanalysis

Utilizing nucleic acid elements for the analysis of nucleic acid targets and non-nucleic acid targets, such as ions, metabolites, proteins, virus, cells and tissues, originates from their abilities in accurate recognition and transduction of target signals as well as versatile reporting methods of output signals. With the help of molecular engineering, different functional nucleic acid probes have been generated for target recognition. Meanwhile, detailed understanding of intra- and inter-molecular interactions between nucleic acids and their targets encouraged further development of nucleic acid-based signal transduction strategies. Furthermore, combined with optical, electrical, magnetic and acoustic technologies, nucleic acid-based bioanalytical strategies can be reported in versatile outputs and applied to different application scenarios. Herein, we will briefly discuss strategies for bioanalysis using nucleic acids in the context of signal recognition, transduction and reporting (Figure 11).

3.2.1 Signal recognition and transduction strategies

As discussed in the previous sections, functional nucleic acids such as aptamers, DNazymes and DNA nanostructures can be used for recognizing targets, either by intramolecular allosteric or intermolecular interactions that can induce changes for signal propagations. Herein, we briefly divided the signal recognition and transduction strategies for nucleic acid-based bioanalysis into two categories: integrated and unintegrated methods.

The integrated method involves single molecule or module that integrates the functions of both target recognition and signal transduction. For instance, the Tan group has reported an aptamer-based molecular probe with intramolecular signal transduction ability [301]. The aptamer

binding to its target molecule will disturb the intramolecular DNA hybridization and induce signal transduction for reporting output. Similar design has been adapted by Wilson *et al.* with tunable control of the thermodynamic and kinetic properties of the aptamer switches for a series of targets [302]. This integrated strategy can ensure the fast activation and quick reporting for bioanalysis due to the two-in-one configuration but may suffer from the disadvantages of less sensitivity owing to no module for signal amplification.

The unintegrated method mainly involves multiple molecules or modules in the nucleic acid-based analysis. For instance, a change induced by the recognition module can propagate to the subsequent module for signal transduction. This strategy allows the enrollment of more functions such as signal amplification and information processing in the bioanalysis, therefore is greatly adopted in different configurations. For example, the Lu group reported a method of using aptamers and DNAzymes as recognition modules and the CRISPR-Cas systems for signal transduction modules with signal amplification ability [303]. Combination of these two modules enables the quick and sensitive detection on a broad range of targets. Han *et al.* developed a DNA displacement reaction-based dynamic system with an aptamer in which the nanostructure can be transformed in response to the epithelial cell adhesion molecule (EpCAM) on live cell membranes and propagate signals through DNA-based signal amplification reactions [304]. Overall, the unintegrated method that involves multiple molecules for signal recognition and transduction is more utilized due to its high flexibility in designing and engineering for different application scenarios.

3.2.2 Signal reporting strategies

As the mostly used methods for detection, optical methods are playing important roles in bioanalysis of various targets. Detection techniques, including fluorescence, colorimetric, and SERS are typical examples. Because nucleic acids can be easily modified with a variety of organic dyes, quantum dots and other fluorescent materials, the fluorophore-based detection methods have been widely demonstrated. For instance, targets such as ions, metabolites, and proteins can be detected by fluorescence due to the binding-induced fluorescence restoration either from proximity change of the fluorophore and quencher pairs or molecular environmental changes of the fluorophores. This strategy has been further expanded to complex biological samples including cells and tissues. You *et al.* used a pair of orthogonal fluorogenic RNA aptamers to quantify the metabolites in live cells [305]. To further fulfill the requirement of deep optical penetration to tissues for *in vivo* probing, nucleic acid probes with Near Infrared (NIR) fluorescence have been developed with the employment of long emission wavelength fluorophores and carbon nanomaterials [306,307]. Until now, the fluorescent reporting strategies still play major roles in nucleic acid-based bioanalysis, especially in the biological and biomedical applications.

In addition, colorimetric and SERS techniques are supplementing the dominant fluorescence-based methods and providing unique advantages for different application scenarios. For example, point of care detection requires readings with either naked eyes or portable equipment, which presents opportunities for colorimetric detection methods. As a kind of indicator, AuNPs can generate colored signals from red to blue on their different aggregation status, which enables convenient colorimetric signal readouts [308]. Similar to the AuNPs, some chemicals and nanomaterials with UV-vis absorption changes upon specific inducement can also be engineered for colorimetric readouts. By coupling functional nucleic acids to these indicators, colorimetric

biosensors with different functions and features can be made and applied for quick and convenient bioanalysis [309]. With the distinct advantages of low background, insensitivity to photobleaching or quenching, single-molecule sensitivity and fingerprint-like spectra, together with the controllable assembly of nanoparticles with functional nucleic acids, SERS can also be used for detection methods in nucleic acid-based bioanalysis. Examples include quantifications of metal ions and proteins *in vitro* as well as tumor markers and metabolites *in situ* [310,311]. Overall, with the development of various technological platforms, the optical reporting strategies will continue play important roles in nucleic acid-based bioanalysis.

Other signal reporting strategies for nucleic acid-based bioanalysis include electrochemical, magnetic and acoustic techniques. Compared to optical-based sensing devices, electrochemical bioanalysis platforms offer the advantages of low cost, simplicity, and the capability of being miniaturized. By integration with electronic devices, the electrochemical nucleic acid-based bioanalysis can provide the possibility of analyzing samples sensitively within short time frames [120]. In addition, more and more magnetic and acoustic techniques have been designed towards the clinical applications due to their intrinsic advantages such as high sensitivity and deep tissue penetration [312,313]. Because nucleic acids can be conjugated to different materials with various properties, they can be used to fabricate different biosensing platforms towards more biological and biomedical applications.

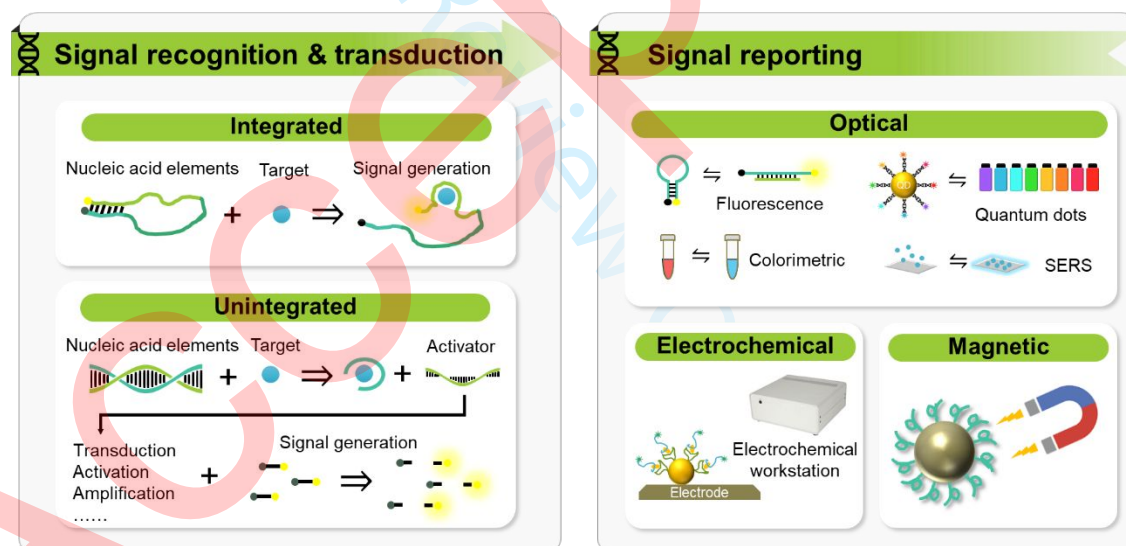


Figure 11. Strategies for nucleic acid-based bioanalysis. The basic modules include signal recognition, transduction and reporting. In the signal recognition and transduction modules, we focused on introducing integrated and unintegrated methods. In the reporting module, optical, electrochemical and magnetic methods for bioanalysis were discussed.

3.3 Representative examples of non-nucleic acid target analysis

Taking advantages of the analysis strategies based on nucleic acid elements, lots of biosensors for various targets have been developed. Herein, several representative examples based on DNA-driven arrangement of nanomaterials are introduced. DNA-driven arrangement of nanomaterials is one effective way to construct functional assemblies, in which nanomaterials can be arranged into specific configurations with strong inter-particle interaction. By choosing different nanomaterials (including AuNPs, gold nanorods (AuNRs), quantum dots, upconversion

nanoparticles (UCNPs), AgNPs) as building blocks, a large number of functional assemblies have been fabricated, such as dimers, trimers, pyramids, core-satellites, and other assemblies. Since these DNA driven assemblies possess striking optical features, they can serve as good candidates for fabricating ultrasensitive sensing platforms to detect many significant analytes, such as ions, proteins, DNAs, RNAs, cells, and so on [314].

Many biosensors with circular dichroism (CD), SERS or fluorescent signal have been fabricated based on the DNA-driven nanostructures. For example, using dopamine aptamer as the linkage, the Au@Ag core-shell nanorod dimers with strong SERS activity were developed for ultrasensitive dopamine detection [315]. In addition, DNA-driven chiral nanostructures were used as chiral biosensors. A chiral AuNP dimer-based sensor for detecting silver ions was developed by Xu's group [316]. Through metal ion-mediated base pairs (cytosine-Ag⁺-cytosine), chiral dimers were assembled by two different-sized AuNPs. The intensity of the plasmonic CD signal was related to the concentrations of the silver ions. Also based on chiral AuNP dimers, a chiroplasmonic sensing platform was applied for detecting adenosine-5'-triphosphate (ATP) [317]. Using DNA aptamers of prostate-specific antigen (PSA), the chiral Au@Ag core-shell nanorod dimers were prepared and used for ultrasensitive PSA detection [318]. Moreover, using metal-ion specific DNAzyme, DNA-bridged chiral core-satellites probe was used for multiple metal ions detection in living cells [22,319,320].

Besides single-mode signal detection, some DNA-driven superstructures can be used for dual-signal detection of some important analytes. For example, the DNA-driven gold-upconversion nanoparticle (AuNP-UCNP) pyramids was used for chiral and fluorescent detection of microRNA-21 (miR-21) in living cells [321]. The Au-UCNP pyramids were composed of two AuNPs and two UCNPs, showing intense plasmonic CD response at 521 nm and strong upconversion luminescence (UCL) signal in the range of 500-600 nm. The Au-UCNP pyramids disassembled in the presence of miR-21, leading to an increase in the luminescence signal and a decrease in the CD signal. In another representative example, a two-signal platform based upon CD and UCL signal, was developed for ultrasensitive DNA detection. Through the DNA-driven self-assemble strategy, three AuNRs and one UCNP were assembled into the propeller-like structure. In the presence of target DNA, the AuNR-UCNP assemblies were destroyed, leading to the decrease of both the CD and UCL signals [322]. Significantly, miRNA-directed intracellular side-by-side self-assembly of AuNR dimers was used for the detection of miRNA in living cells. The more miRNAs existed, the more AuNR dimers formed. The CD response and SERS intensity were proportional to the amount of target miRNAs. The linear range of the CD signal was 0.11-42.23 fmol/10 μ g RNA, with a LOD of 0.081 fmol/10 μ g RNA, while the SERS activity showed the same detection range with a LOD of 1.12 fmol/10 μ g RNA [212].

It is a great challenge for simultaneous detection of different types of biomolecules (i.e., nucleic acids and proteins). With ingenious design of DNA structure, a unique and universal strategy based on dual-model optical sensor (SERS and UCL) was proposed for quantification of miR-21 and telomerase in living cells [323]. In the presence of miR-21 and telomerase, the hybridization of miR-21 with a MB causes the separation of 3, 3'-diethylthiocarbamyl cyanine iodide-modified AuNR dimers, resulting in a decrease in SERS signal. At the same time, the target telomerase elongates the telomerase primer strands, which is followed by substitutional hybridization and release of UCNPs, resulting in an increase in UCL signal (Figure 12(a)). In another example, the

[illegible]

Figure 12. Representative examples of non-nucleic acid target analysis. (a) Schematic of Au NR dimer-UCNP core-satellite nanostructures used for the simultaneous analysis of intracellular miR-21 and telomerase [210], Copyright 2017 American Chemical Society. (b) GO-Au assemblies strategy for EpCAM (probe Ep) and miR-21 detection (probe mR) [210], Copyright 2017 WILEY-VCH.

4. Device development for nucleic acid analysis

As an essential biological molecule, nucleic acids participate in many physiological and pathological processes [324]. Aberrant expression and mutations of nucleic acid sequences may lead to occurrence of various diseases, such as cancer [325,326]. Thus, highly efficient detection and analysis of target nucleic acids are of great importance. One of the key demands for accurate nucleic acid analysis is to develop highly sensitive and precise analytical devices that can provide valid information and guidance for molecular diagnosis and disease treatments [327]. Different from bulk and large-scale instruments, miniaturized and portable devices with low cost, easy operation, high-throughput and multiplex performance are rather desirable to practical use, especially for clinical applications [328]. Aiming at these advantages, a wide range of devices have been extensively exploited as the promising tools for efficient nucleic acid analysis [329,330]. In this section, we highlight various miniaturized devices using for nucleic acid analysis and detection *in vitro*, including PCR detection systems, microfluidics, handheld instruments, and paper-based systems.

4.1 PCR device

PCR is a molecular biology technique used to amplify specific DNA fragments [331]. Benefiting from the unique properties of high sensitivity, versatility, rapidity and easy to manipulate, PCR has been widely applied for molecular diagnosis of genetic disease, pathogens and oncogene, especially for the fields of nucleic acid analysis [332-334]. To meet the rapid advances of PCR technique and molecular analysis, various automated and functional PCR-based devices have been gradually developed for different analytical requirements [335,336]. Conventional PCR thermal cyclers generally focus on the temperature control and nucleic acid duplication during amplification processes [337]. The drawbacks of large instrument size with tedious operation time and high-power consumption hamper their practical applications. With the rapid development of automation and integration technology, more intelligent, miniaturized and precise PCR devices have been extensively exploited [338,339]. For instance, Helb *et al.* used a simple cartridge system with real-time PCR, named Cepheid GeneXpert System, to detect *Mycobacterium tuberculosis* and rifampin resistance [340]. The limit of detection of the *Mycobacterium tuberculosis* DNA was 4.5 genomes per reaction. Hatch *et al.* reported a low power, high-throughput and portable qPCR device with continuous flow thermal cycling manners that could be used for multiple analyte detections (Figure 13(a)) [341]. Only 0.1-10 μ L droplet samples were needed to realize the high PCR amplification efficiency and sensitive fluorescence detection. The PCR efficiency of this device could be reached within the range of 90-110%, and the limit of detection was 10 copies per reaction. Li *et al.* developed a photonic-PCR system that integrated Fe_3O_4 nanoclusters for effective photothermal cycle modulation with a battery-powered device for successful data transfer (Figure 13(b)) [342]. The system was energy efficient and easy establishment, which efficaciously applied for the detection of defective mtDNA in mitochondrial

diseases with low initial template concentration (50 pg-500 ng) and highly reasonable linearity. Despite the significant improvements of PCR devices, the highly sensitive reaction condition and the rigorous temperature calibration still remain big obstacles for more precise and controllable PCR-based diagnosis. Based on this circumstance, Adams *et al.* constructed a prototype adaptive PCR instrument focusing on the dynamically sensing the hybridization state during the PCR process instead of the temperature monitoring (Figure 13(c)) [343]. This system has been proved more robust and simpler, and it could overcome the limitation of strict temperature calibration in some traditional PCR instruments. Additionally, the occurrence of dPCR with absolute quantification also largely promotes the advance of PCR devices [344-346]. Gou *et al.* constructed a handheld smartphone-based dPCR device for the detection of the plasmid DNA with the human 18S ribosomal RNA gene fragment [347]. The device functions could be automatically controlled by a custom software, and the accurate detection could reach down to 10 copies. Moreover, Zhou *et al.* developed a highly integrated real-time dPCR device that constructed based on off-the-shelf electronics and 3D printing technology [348]. This dPCR device was successfully applied to the plasmid DNA targets with enhanced accuracy and could overcome the misclassification of positive partitions in some classical dPCR devices. Overall, PCR is still a promising development direction in the application of nucleic acid analysis. By combining with advanced microanalysis technology and molecular biotechnology, more integrated and smarter PCR devices with higher accuracy and sensitivity will be widely exploited and applied.

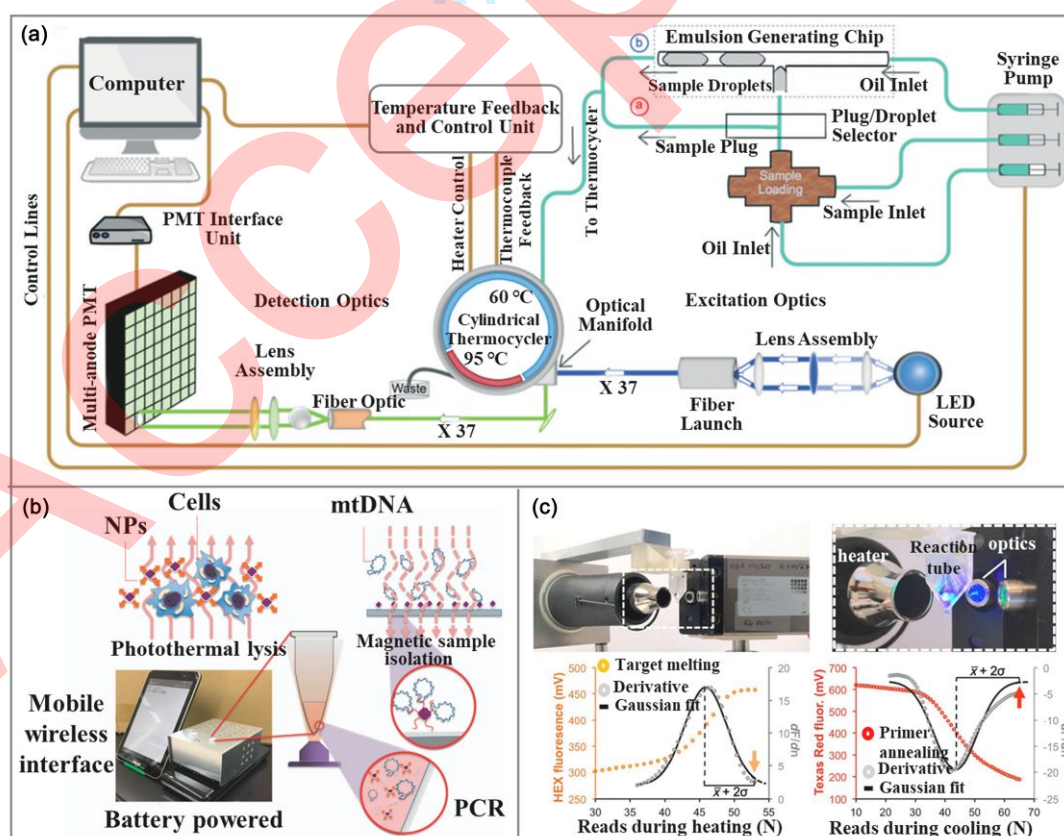


Figure 13. PCR devices for nucleic acid analysis. (a) Schematic illustration of the portable real-time PCR device with continuous flow thermal cycling for multiple DNA sample analysis [341], Copyright 2014 Royal Society of Chemistry. (b) Schematic illustration of the handheld qPCR device integrated with magnetic nanoparticles for effective target DNA detection [342],

Copyright 2016 Nature Publishing Group. (c) Schematic illustration of the adaptive PCR device to dynamically control and monitor the DNA replication processes [343], Copyright 2017 American Chemical Society.

4.2 Microfluidic device

Microfluidics featuring at reduced sample consumption and shortened reaction time has been extensively studied for nucleic acid analysis, because of its capability to integrate different operation units, such as sample pretreatment, nucleic acid extraction and amplification, and signal detection into a single microfluidic device [349-352]. A microfluidic cassette was designed for nucleic acid detection of Zika Virus [353]. The sensitivity of this platform was less than 5 plaque-forming units (PFU) per sample and the total detection time was less than 40 min. A self-powered integrated microfluidic chip was reported for rapid digital detection of nucleic acids from human blood sample (Figure 14(a)) [354]. This microfluidic chip powered by a vacuum battery was prepatterned with the amplification initiator MgOAC. After mixing with reagents, the blood sample was introduced into microfluidic chip and then separated autonomously into microwells for isothermal recombinase polymerase amplification (RPA) reaction. This microfluidic chip enabled detection of methicillin-resistant *Staphylococcus aureus* DNA (10 to 10⁵ copies per microliter) within 30 min. In a recent work, a hand-powered centrifugal microfluidic platform was developed for sample-to-answer detection of pathogens (Figure 14(b)) [355]. Through a simple pull-out operation of the customized centrifuge, the pathogen sample was rapidly mixed with the preloaded reagents inside the microfluidic disc. After on-disc LAMP at 65 °C heated by pocket warmers, the presence of target pathogens can be observed by visible fluorescence. This microfluidic platform allowed for simultaneous detection of six pathogens with the sensitivity of 2×10^2 cells per μL . For diagnostics of miRNA in serum, a CRISPR/Cas13a-based electrochemical microfluidic biosensor was designed (Figure 14(c)) [356]. This amplification-free platform showed a limit of detection of 10 pM, and the total process time was less than 4 h. A centrifugal microfluidic platform with all reagents prestored was reported for nucleic acid analysis of pathogen by qRT-PCR [357]. The whole detection procedure was carried out within a 2 kg portable instrumentation and the total time was less than 3.5 h. Self-contained microfluidic platforms integrated with a variety of operations and functions, show great potential for nucleic acid diagnostics in POC formats.

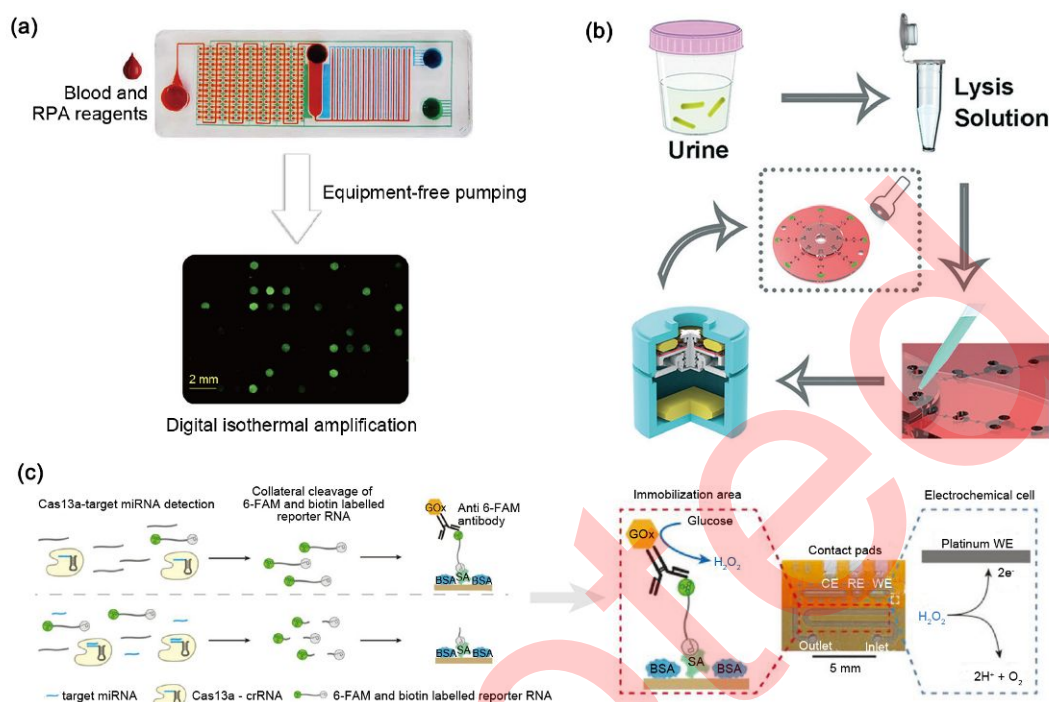


Figure 14. Microfluidic devices for nucleic acid analysis. (a) Self-powered integrated microfluidic chip for RPA-based quantitative detection of DNA (Yeh, *et al.*, 2017), Copyright 2017 American Association for the Advancement of Science. (b) Schematic illustration of sample-to-answer nucleic acid detection by the hand-powered centrifugal microfluidic platform [355], Copyright 2018 Royal Society of Chemistry. (c) CRISPR/Cas13a-powered electrochemical microfluidic platform for miRNA diagnostics (Bruch, *et al.*, 2019), Copyright 2019 Wiley-VCH.

4.3 Handheld device

Many commercial and handheld devices, such as glucometer, pressuremeter, thermometer and pH meter, are successfully available to the public with easy use and excellent sensitivity. Recently, by combining these handheld devices with biological recognition and amplifying transducer components, portable biosensors for various targets detection with high sensitivity have been exploited, such as nucleic acids [358]. Glucometer was the first portable device being used for non-glucose target detection [359,360]. As shown in Figure 15(a), Xu *et al.* used DNA sandwich hybridization happened between the HIV target DNA and the capture probes as well as the signal probes to introduce multi-invertase conjugated nanoparticles to the surface, which can enzymatically convert sucrose to glucose for signal amplification and glucometer monitoring [361]. Si *et al.* reported an oligonucleotide cross-linked hydrogel for recognition and quantification of miRNA with glucometer [362]. In their work, the target miRNA was the essential component to form DNAzyme that can cleave the crosslinkers of hydrogel to destroy hydrogel and release encapsulated amylase, which can further catalyze the hydrolysis of amylose to produce a large amount of glucose for glucometer readout. The dual amplification by DNAzyme and amylase resulted in high sensitivity that as low as 0.325 fmol miRNA can be portably detected by glucometer. Pressuremeter is another user-friendly and portable device with wide dynamic range (0-3000 kPa) and low detection sensitivity (0.01 kPa) [363]. Shi *et al.* designed target miRNA induced cyclic strand displacement to enrich platinum nanoparticles onto

the surface of magnetic beads, which can catalytically decompose H_2O_2 for the generation of O_2 to induce pressure change with pressuremeter readout (Figure 15(b)) [364]. Toumazou *et al.* has found out that the released hydrogen ions during nucleotide incorporated DNA amplification can be directly measured to quantify target sequence [365]. Using this strategy, Yuan and coworkers simply used the pH meter to measure the hydrogen ions generated by loop mediated isothermal amplification for the facile and sensitive detection of *Nosema bombycis* genomic DNA (Figure 15(c)) [366]. Recently, Li group reported the use of a common thermometer for visual and quantitative genetic detection [367]. As shown in Figure 15(d), single-stranded DNA on AuNPs would be deprived because of the hybridization with target DNA, resulting in AuNP aggregation upon the addition of salt. While being irradiated by NIR laser, temperature was obviously elevated in the aggregated AuNP suspension. As high as 18 °C temperature change can be readout by thermometer and the detection limit of 0.28 nM was achieved without amplification. Besides, other handheld devices such as electronic balance [368], digital multimeter [369], uric acid meter [370], etc. could also be used for nucleic acid analysis with appropriate signal transduction and signal amplification mechanism. The use of these handheld devices that are widely applied in daily life, opens a new door for simple, cost-effective, user-friendly and portable POC testing of nucleic acids with high specificity and sensitivity and without external bulky instrumentation.

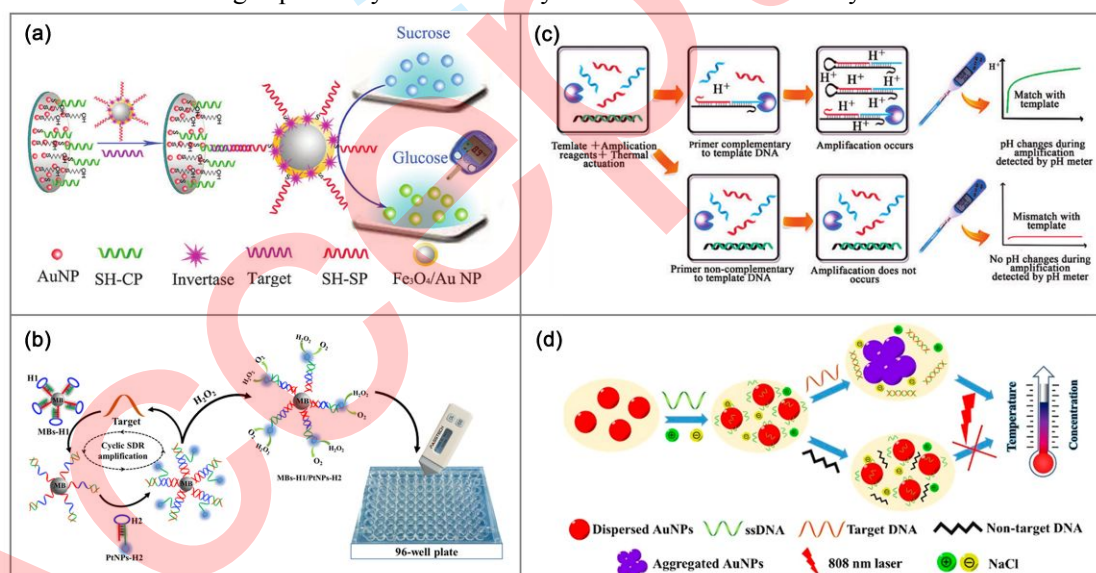


Figure 15. (a) Schematic presentation for sensitive POC detection of HIV DNA fragments using glucometer [361], Copyright 2012 Royal Society of Chemistry. (b) Schematic illustration of gas pressure-based POC assay for sensitive detection of miRNA with pressuremeter [364], Copyright 2018 American Chemical Society. (c) Schematic illustration of the amplified detection of target DNA based on pH meter [366], Copyright 2014 Royal Society of Chemistry. (d) Schematic illustration of AuNP aggregation-induced photothermal biosensing of target DNA using thermometer [367], Copyright 2020 American Chemical Society.

4.4 Paper-based device

Traditionally, paper has been extensively used for printing and writing owing to its low price and high yield. Beyond that, because of the fibrous and porous structure, high surface-to-volume ratio and hydrophilicity [371], paper has been also exploited as a substrate material for metal detection [372], paper chromatography [373] and bioanalysis system [373-379]. Using printing

techniques, such as inkjet printing [380], wax printing [381] and screen printing [382], paper has been used to construct different analytical devices by modifying with small molecules, nucleic acid, antibodies or other proteins (Figure 16) [377,380,383]. Thus, paper-based analytical devices (PADs) as promising technologies provide alternative platforms to develop low-cost, portable, rapid and disposable biosensors for POC diagnostic [384-388]. With the development of nucleic acid technology, a broad spectrum of nucleic acid-based PADs has been exploited to detect a wide variety of analysts by electrochemical, colorimetric, chemiluminescent or fluorimetric readouts.

Electrochemical PAD biosensors are constructed often through wax-printing or screen printing [378,379,389-391]. In 2009, the first electrochemical PAD was developed by Dungchai *et al.* [392]. Since then, electrochemical PADs aroused wide interest because of easy use, uncomplicated equipment, cost-efficient and rapid analysis, portable potentiostats, renewable sensing interface and well-known electrochemical signaling methods [393]. The fabrication processes are divided into three steps roughly: Firstly, suitable electrodes were chosen as reference electrode, working electrode and counter electrode, respectively. Secondly, electrodes are printed onto paper by using printing techniques. Thirdly, nucleic acids are modified on working electrodes through chemical reaction, generating sensing interface. Until now, a wide range of electrochemical nucleic acid-based PADs have been developed [394].

Benefiting from the simplicity, sensitivity and low cost, colorimetry detection technique is considered to be suitable for integrating with PADs [395]. Colorimetric assays in PADs are accomplished usually depending on the aggregation of noble nanoparticles [395,396] or the oxidation of chromogenic substrate [396-400]. The target-stimuli signal, color change, can be visualized by naked eyes and the detection zone images can be collected by camera phone or portable scanner and then transformed into quantitative information by software [394,401-403]. On account of the optical properties [404] and easy modification with bio-recognition elements [405,406], gold nanoparticle becomes one of the most popular nanoparticles in colorimetric assays. For instance, a colorimetric assay was performed on paper-based analytical device for the detection of prostate specific antigen in human serum taking advantages of AuNPs modified with nucleic acid and horseradish peroxidase capable of oxidizing TMB with the assistance of hydrogen peroxide [400].

Based on fluorimetric/chemiluminescent molecules and nanoparticles (e.g., fluorophores and quantum dots), paper-based DNA biosensors also are often constructed for the detection of different analysts, such as metal ions, DNAs and cancer cells [407-412]. For example, Wang and partners developed a chemiluminescent PADs to distinguish target DNA from single-mismatched one by employing carbon dots dotted nanoporous gold as amplification signal label [412]. In addition, dual output signals (e.g., electrochemical/colorimetric signals) paper-based biosensors are also proposed [123,410,413,414].

Various nucleic acid-mediated reactions have been exploited in PADs, including simple DNA hybridization [378,379,389,395,409] and complex reactions, such as PCR [415], RPA [398,416], RCA [396,399,417], and SDA [418,419]. The sensing interfaces are composed of oligonucleotide monolayers [379,412,420], composite multilayer [378,409] and 3D nucleic acid materials [399,410] or oligonucleotide-containing heterogeneous nanostructures [123]. The analysts include small molecules [375,421], miRNAs [378], DNA strands [123,379,412,420], proteins [375,379,391,422], exosomes [409], cancer cells [422], papillomavirus [389], pathogens [408] and so on, implying the great potential of nucleic acid-based PADs in enormous applications.

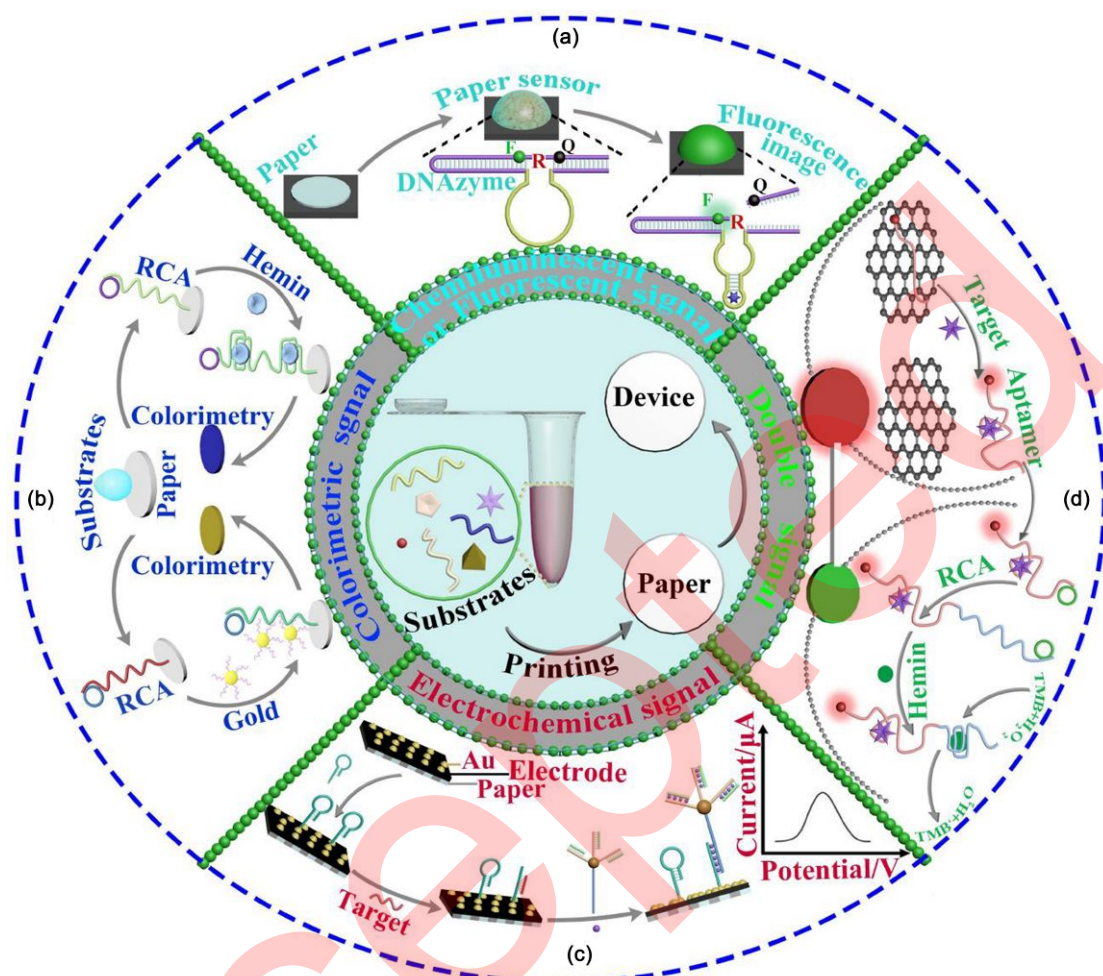


Figure 16. The schematic diagram of paper-based devices. (a) Paper-based chemiluminescent or fluorescent devices based on signaling nanoparticles (e.g., fluorophores and quantum dots) and auxiliary molecules. (b) Paper-based colorimetric device via utilizing the aggregation of noble nanoparticles (such as gold nanoparticle) or the oxidation of chromogenic substrates. (c) Paper-based electrochemistry devices by printing working electrodes with nucleic acids on paper. (d) Paper-based devices with dual output signals (e.g., fluorescent/colorimetric signals).

5. Applications of nucleic acid analysis

Over the past decades, nucleic acid analysis has experienced rapid advances with new analysis techniques, devices and kits. Owing to its merits of sensitivity and specificity, it has been widely applied in clinical translation, environmental analysis, food safety, forensic analysis and so on.

5.1 Clinical translation

Nucleic acid-based molecular diagnostics is a collection of techniques used to analyze nucleic acid markers of diseases, promoting the development of personalized medicine [423]. It has been widely applied in clinical practice for the screening, diagnosis, risk assessment, and treatment guidance of diseases, including cancer, congenital anomaly, infectious disease, etc. Thereinto, many techniques have achieved clinical translation with commercial devices and assay kits [424]. For precision diagnosis and therapy of cancer, the detection of DNA mutation, DNA methylation, and miRNA, as well as the expression analysis of key genes from tumor-biopsy specimens has

been performed, such as epidermal growth factor receptor (EGFR) mutation, human epidermal growth factor receptor 2 (HER2) amplification, etc [425-427]. For prenatal testing, nucleic acids analysis of fetal cells from amniocentesis and chorionic villus sampling enables to detect problems such as chromosome abnormalities and gene mutations, serving as a powerful method to control genetic diseases and birth defect [428]. More importantly, the rapid advance of PCR techniques and NGS together with liquid biopsy allows sensitive and reliable molecular diagnosis of tumor/fetal-derived circulating targets, including circulating tumor/fetal cells (Figure 17(a)), cell free circulating DNA (Figure 17(b)), circulating miRNAs, extracellular vesicles, tumor-educated platelets, etc [429-435]. These approaches offer comprehensive and timely information for personalized anti-cancer therapy and noninvasive prenatal testing (NIPT).

Pathogen infection is a common health problem worldwide. Especially, the pandemic of corona virus-based COVID-19 since the end of 2019 poses great threats to the public health. Thus, it is critically important to develop rapid, sensitive, and reliable methods to detect and identify pathogens for adopting effective therapy and controlling epidemic. Nucleic acid analysis is popularly utilized for infection diagnosis clinically, and also serves as a gold standard method for pathogen identification [436]. Nucleic acid amplification analysis with exquisite sensitivity and specificity allows for rapid and accurate detection of pathogens, free of time-consuming culture (Figure 17(c)) [437]. Multiplex PCR and various hybridization-based biosensors afford multiplex pathogen analysis [438,439]. Besides, high-throughput sequencing enables identification of unknown pathogens and determination of drug resistance-related genes or gene mutations [440]. Moreover, sequence data can be used to reconstruct the evolution of pathogens and predict future spread for better intervention [441]. Considering that pathogen infection is still the leading causes of death in low-income countries, more efforts should be devoted into the development and clinical translation of reagents and instruments of nucleic acid-based point-of-care testing (POCT).

Besides taking nucleic acids as analysis targets, functional nucleic acids can be utilized as recognition tools and drugs for clinical diagnosis and therapy [442,443]. For example, numerous aptamer-based biosensors have been developed for sensitive detection of disease biomarkers and pathogens [444]. Pegaptanib, as a pegylated anti-vascular endothelial growth factor (VEGF) aptamer, was approved by U.S. Food and Drug Administration (FDA) in 2004 for the treatment of age-related macular degeneration [445]. Very recently, several aptamers targeting proteins of 2019-nCoV have been reported [446-448].

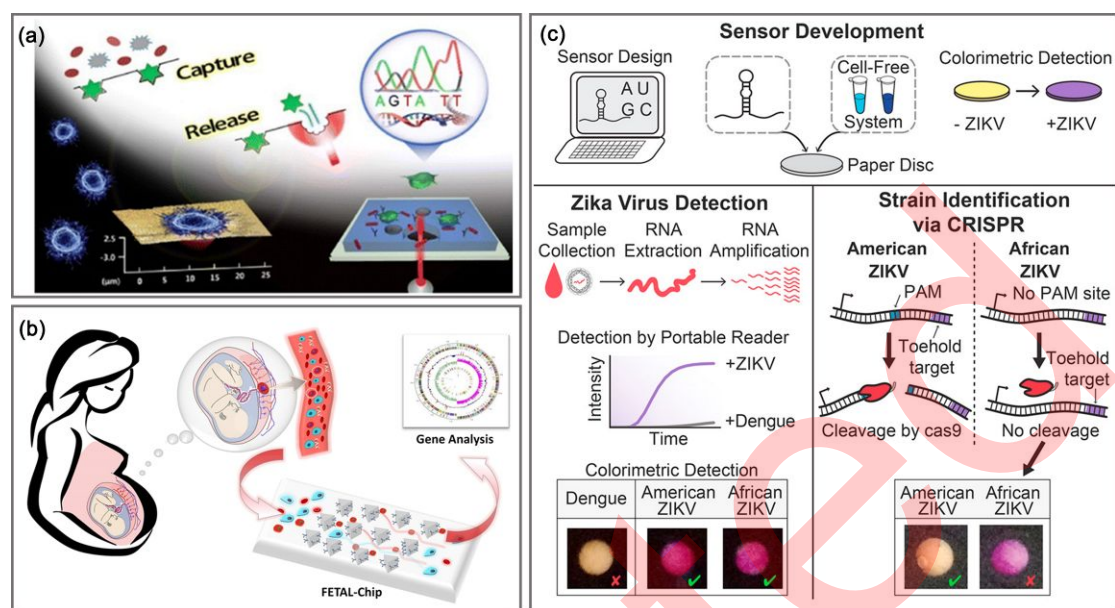


Figure 17. Clinical translation of nucleic acid analysis. (a) Gene mutation analysis of circulating tumor cells [434], Copyright 2016 American Chemical Society. (b) Gene analysis of circulating fetal cells for NIPT [435], Copyright 2018 The Royal Society of Chemistry. (c) Paper-based sensors to detect RNA genome of the Zika virus [437], Copyright 2016 Elsevier.

5.2 Environmental analysis

Earth's biodiversity is undergoing continuous decline, which represents a major environmental crisis. Coupled with sequencing technique, environmental DNA, defined as genetic material from environmental samples, is considered as potential candidate for biodiversity monitoring (Figure 18(a)) [449]. Thereinto, environmental microbiology attracts the most attention due to the vital role of microorganisms in regulating ecosystem system. Metagenomics sequencing and 16S rRNA gene sequencing are capable of characterizing the diversity and abundance of microbial communities [450,451]. Thus, influence factors on microbial communities can be evaluated for pollution abatement and ecosystem management, such as environmental pollution, climate change, introduced species, human activities [452]. Additionally, nucleic acid analysis techniques facilitate the identification and isolation of microorganisms with specific phenotypes, such as mercury resistant bacteria in contaminated environments [453]. Therefore, nucleic acid analysis is acting as a powerful means in environmental monitoring and management.

The increasing presence of harmful pollutants in the environment affects human health, and calls for analytical methods for pollution monitoring. Nucleic acid-based biosensors with merits of miniaturization, portability, and integration open new avenues for the detection of environmental pollution and toxicity (Figure 18(b)). On the one hand, taking target nucleic acid sequences as analytes, hybridization-based biosensors enable to detect pathogens, harmful algae, and genetically-modified plants in environmental samples [454]. On the other hand, taking functional nucleic acids as recognition elements, including aptamer, DNAzyme, and allosteric DNAzyme, numerous biosensors have been developed for convenient and rapid detection of metal ions, chemical residue, etc [455,456]. Besides, DNA damage caused by pollutants (e. g., heavy metals, pesticide residue, etc.) could be detected with electrochemical DNA-based biosensors, allowing for toxicity screening and environmental monitoring [457].

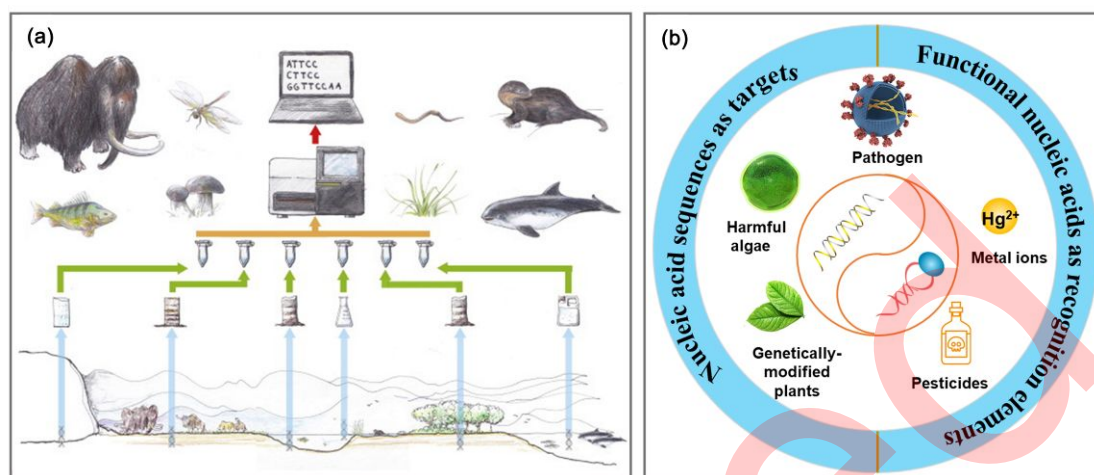


Figure 18. Nucleic acid-based environmental analysis. (a) The overall workflow for environmental DNA analysis [446], Copyright 2015 Elsevier. (b) Nucleic acid-based biosensors for the detection of environmental pollution and toxicity.

5.3 Food analysis

Food analysis is of great significance to prevent foodborne diseases which are mainly originated from foodborne contaminants including chemical and biological hazards. The above-mentioned nucleic acid-based pathogen analysis techniques are also widely used to detect and identify foodborne pathogens and to study food microbial ecology [458,459]. Functional nucleic acids, coupled with novel transducers and nanoprobe, offer a variety of biosensors for foodborne contaminant detection [460-462]. These biosensors have achieved rapid and sensitive detection of pathogens (Figure 19(a)) [463], mycotoxins (Figure 19(b)) [464,465], allergens [468], pesticides [467], heavy metal ions [468], illegal additives [469], with a great application potential in POCT. In the future, the development of low-cost, rapid and miniaturized analytical technologies will still be the important mainstream in this field.

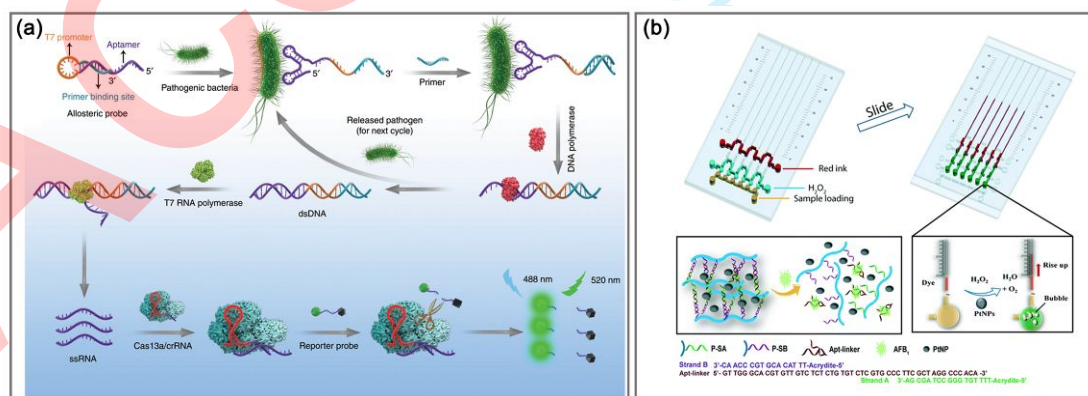


Figure 19. Nucleic acid-based biosensors for food analysis. (a) Aptamer-based allosteric probes for the detection of foodborne pathogens using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas13a [463], Copyright 2020 Nature Publishing Group. (b) Target-responsive aptamer hydrogel for portable visual quantitative detection of aflatoxin B1 using a distance-readout microfluidic chip [465], Copyright 2016 The Royal Society of Chemistry.

5.4 Forensic analysis

DNA typing has become an indispensable tool for individual identification in forensic analysis, including homicides, sexual assaults, missing persons, parental testing, etc. Short tandem repeat (STR) sequences are most widely utilized for forensic human identification [470]. The genetic markers located on the Y chromosome can provide male specific information for investigating sexual assault and unestablished paternity [471]. As for degraded or low template samples, single nucleotide polymorphisms (SNPs) and mtDNA could provide additional genetic information to augment forensic analysis. Additionally, mRNA signatures have been widely applied in many forensic laboratories for body fluid identification [472]. The RNA decay analysis facilitates the determination of the post-mortem interval and the age of biological stains [473]. MiRNA is more stable and has greater discriminatory potential than mRNA, and its profiling is attracting more attention in forensic science [474]. Additionally, sequencing-based microbial forensics contributes to human identification, geolocation, postmortem interval estimation and bioterrorism defense [475]. Notably, microfluidic devices have been designed for forensic analysis with merits of reduced risk of contamination, shortened analysis time and supporting POCT [476].

5.5 Security

Information security is one of the vital issues in modern society, which requires cryptography schemes to protect messages for secure communication. With the advantages of vast storage density and massive parallelism capacity, nucleic acid molecules have attracted considerable attention in the field of information security [477,478]. DNA cryptography is emerged for encryption, authentication and signature by harnessing nucleic acids as informational and computational carriers [479,480]. DNA with high memory density makes it possible for one-time-pads (OTP) cipher cryptosystem which is considered as the most secure strategy of private key encryption. Many DNA-based encryption methods have been designed for OTP, and PCR and DNA chip techniques are the most prominent choices [481,482]. DNA chip techniques enable to encrypt both textual data and two-dimension images [483]. Besides sequence information, structural pattern of DNA nanostructures provide an alternative strategy for cryptography (Figure 20) [484]. Nucleic acid-based information steganography can hide secret information as nucleic acid strands for transmission in various carriers, such as DNA microdots, genetic codon and living organisms [480,485]. Although significant and promising, nucleic acid-based information security still stay in a theoretical stage, and intensive efforts should be made in this field to improve the accuracy, efficiency, and robustness.

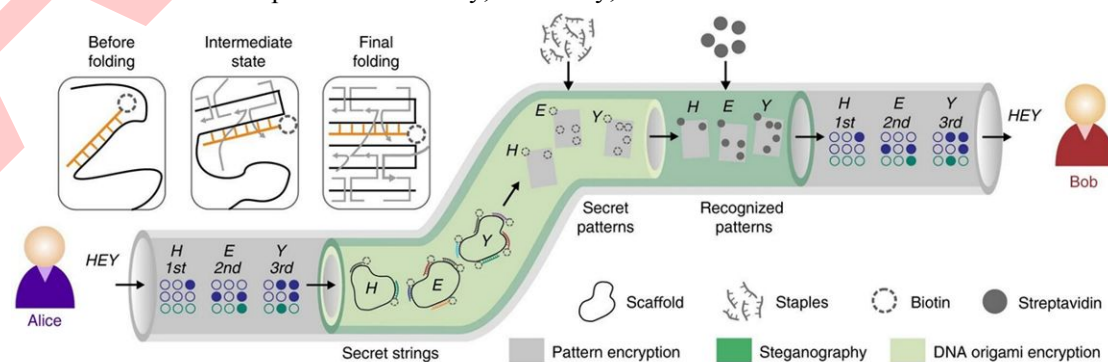


Figure 20. DNA origami-based cryptography with multi-level protection for secure communication [255], Copyright 2019 Nature Publishing Group.

6. Conclusions

Nucleic acids analysis holds great promise as a bioanalytical paradigm for various applications in biology, chemistry and clinics. Recent developments on the analysis of nucleic acids including *in-vitro* analysis based on hybridization, target amplification and sequencing have led to applications for intracellular and *in-vivo* analysis. The use of nucleic acids as recognition elements (e.g., aptamers, DNAzymes) and engineering tools (e.g., DNA/RNA nanostructures) in bioanalysis is also of particular focus to bring nucleic acids into the analysis of a broad spectrum of targets including ions, metabolites, proteins, exosomes, even whole cells. Furthermore, the incorporation of nucleic acids analysis in electronic / mechanic devices opens new opportunities for translating laboratory techniques to real-world applications. Especially, applications of nucleic acids analysis and nucleic acids-based analysis in clinical diagnostics, food analysis, environmental analysis, forensic analysis and homeland security have shown great potential. We envision that the field of nucleic acids analysis will continue to grow benefiting from coupling of integrated and miniaturized biosensing devices, which can expand the application of nucleic acids-based sensors to enable the real-time study of biological process and to minimize the invasion for biomedical applications. The improvement of the aptamer selection strategy will expand the range of detectable targets with high specificity and selectivity. Novel signal transduction and signal amplification strategies may improve the detection sensitivity down to single cells, single particles and single molecules. The development of the high-throughput of biosensors will further increase the capability of biosensors to be coupled with big data analysis to accelerate the development of precision medicine.

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Conflict of interest The authors declare that they have no conflict of interest.

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