Chem Soc Rev

The development of nanostructure assisted isothermal amplification in biosensors

Journal:	*Chemical Society Reviews*
Manuscript ID	CS-REV-10-2015-000819.R1
Article Type:	Review Article
Date Submitted by the Author:	28-Dec-2015
Complete List of Authors:	duan, ruixue; Huazhong University of Science and Technology Lou, Xiaoding; Huazhong University of Science and Technology, xia, fan; Huazhong University of Science and Technology,

The development of nanostructure assisted isothermal amplification in biosensors

Ruixue Duan, Xiaoding Lou*, Fan Xia*

Key laboratory of Material Chemistry for Energy Conversion and Storage, Ministry of Education, School of Chemistry and Chemical Engineering, National Engineering Research Center for Nanomedicine, Huazhong University of Science and Technology (HUST), Wuhan, China.

E-mail: xiafan@hust.edu.cn

louxiaoding@hust.edu.cn

Abstract

Developing simple and inexpensive methods to ultrasensitively detect biomarkers is important for medical diagnosis, food and environment security. In recent years, isothermal amplifications with sensitivity, high speed, specificity, accuracy, and automation have been designed based on interdisciplinary approaches among chemistry, biology, and material sciences. In this article, we summarize the advances of nanostructure assisted isothermal amplification in past two decades for detection of the commercial biomarkers, or biomarkers extracted from cultured cells or patient samples. This article has been divided into three parts according to the ratio of target-to-signal probe in the detection strategy, that is N:N amplification ratio, 1:N amplification ratio, and the 1:N^2 amplification ratio.

1. Introduction

Nanostructures with different dimensions are used in constructing high efficient biosensors (Figure 1). DNA linear probe (0D) is first developed to detect DNA via Watson-Crick base pairing. However, it needs wash steps to remove DNA linear probes nonspecifically bound to surface and unhybridized target DNA, reducing the detection speed and decreasing sensitivity [1-3]. The molecular beacon (MB) (0D) (Figure 2A, 2B) developed as early as 1996 has been popular used in biosensors because it can report the presence of the target by the specific target-triggered change in its conformation [3-11]. The sandwich structure (0D) (Figure 2C), recognition probe-target-signal probe, is also a mainstay in biomarkers detection with high specificity because it requires the simultaneously binding of two recognition elements and target [12-15]. Recently, Fan et al. designed a tetrahedron-structured DNA probe (Figure 2D) that consists of 4 single stranded DNAs and can be assembled on surface in a single step with well-controlled spacing and high stability, greatly reducing the background noise [16-18]. Although the MB, sandwich structure, and tetrahedron DNA probe play important roles in enhancing specificity, the detection only based on those DNA nanostructure assisted non-amplification is still limited by sensitivity because the ratio of target-to-signal is 1:1. Polymerase chain reaction (PCR) is the most widely used exponential amplification method for biomarkers detections. However, thermal cycling instrumentation, easy contamination, and sophisticated optimization limit its utility. Furthermore, it is not suitable to adjust the temperature in vivo analysis, restricting

the application of PCR.

Recently, isothermal amplification that proceeds at constant temperature for amplified detection of biomarkers has emerged as an alternative to PCR due to its easy operation, quick results, PCR-like sensitivity, low cost and energy efficiency. The isothermal amplification can be broadly divided into enzymatic amplification and enzyme-free amplification. Enzyme assisted isothermal amplification techniques always use the above mentioned DNA structures and employ strand displacement polymerase [19-23], exonuclease [24-32], nicking enzyme [33-37], or duplex-specific nuclease [38-41] to recycle the targets, increasing the detection sensitivity quickly [42]. While the enzyme-free isothermal amplification strategies achieve attractive sensitivity and is widely used in vivo detection by means of supersandwich structure (1D) [43-50], biobarcode probe (3D) [51-55], HCR (hybridization chain reaction, 1D) [56-60], and hairpin DNA cascade amplification (0D) [61-63]. Although the isothermal nature enables strategy time-saving and versatile, it also results in non-specific reaction and high background noise.

Over the past decade, non-DNA nanostructures have attracted much attention in bioanalytical and biomedical applications [64-65]. For instance, Quantum dots (0D) have been widely used because of its broad excitation, size-tunable photoluminescence spectra, and narrow emission bandwidth compared to traditional organic dyes [66-67]. The plasmonic gold nanoparticle (GNP) ($d > 3.5$ nm) (0D) is well known for its high extinction coefficients, distance-dependent optical properties, and ultrahigh fluorescence quenching ability, which makes it one of

promising materials [68-71]. Graphene oxide (GO) (2D), a monolayer of graphite sheet, is also one of the most promising materials for biosensors due to its highly conductive, transparent, bendable, functionalizable surfaces, the property of fluorescence quenching, the ability to bind single strand DNA and protect the single strand DNA from nuclease [72-83]. Therefore, the combination of enzyme assisted isothermal amplification with those non-DNA nanostructures contributes to decrease background, increase sensitivity, simplify operation, and boost accuracy. Additionally, those non-DNA nanostructures have big specific surface areas, which favors high-throughput automation detection.

Here we describe the recent development of isothermal amplification technology based on DNA-nanostructures and non-DNA nanostructures. Firstly, we discuss the improved strategy to enhance the specificity and sensitivity based on supersandwich structure, in which the ratio of target to signal probe is N:N. It should be noted that multiple signal probes are assembled via multiple target DNAs, and then acts as a whole in this strategy, greatly enhancing sensitivity and specificity compared to that of traditional 1:1 detection strategy. Secondly, we summarize the various isothermal linear amplification methods lie on QDs, GO, GNPs, biobarcode amplification, hairpin DNA cascade amplification, and HCR, in which the amplification ratio is 1:N. Finally, we report the most recent quadratic amplification with ultra-high sensitivity by using functional MB, improved linear probe, and even non-DNA nanostructures, in which the amplification ratio is $1:N^2$.

2. Detection strategy based on the ratio of target to signal is N:N

Sandwich structure, a DNA nanostructure that attempts to reduce background noise from non-specific hybridization, plays an important role in analysis of DNA and protein. However, the intrinsic limitation on sensitivity restricts its application because a DNA target only hybridizes to a single signal probe. In response, our group reported a supersandwich strategy in which the ratio of target to signal probe is N:N, greatly enhancing the sensitivity and selectivity compared to the traditional sandwich assay (Figure 3) [45]. By rationally design the sequence of signal probe, a signal probe can hybridize with two regions of a target DNA. Therefore, in our new strategy, the DNA target hybridized to the signal probe and the sticky end of the signal probe hybridized with another target, forming a supersandwich structure containing multiple labels, realizing signal amplification and improving the detection sensitivity. Recently, the supersandwich strategy was also used to detect other molecules, such as protein and ions [46-47]. For example, Li et al. reported an electrochemical strategy for detection of platelet-derived growth factor-BB (PDGF-BB) by fabricating an aptamer-protein nanowire [46]. The amplified signal can be detected via a large amount of redox species ($[Ru(NH_3)_6]^{3+}$) attached by the nanowire, and the detection limit as low as 100 fM can be obtained. In another study, Xu et al. developed an electrochemiluminescence assay for Hg^{2+} detection based on fabricating T–Hg^{2+}–T assisted supersandwich structure [47]. This strategy exhibited high sensitivity and selectivity, and the detection limit can be as low as 0.25 nM. It is worth noted that the N:N strategy occurred on electrode surface is not equivalent to

1:1. For the strategy in which the ratio of target-to-probe is N:N, a large number of signal probes are integrated into a coherent whole and attached onto the surface, enormously enhancing the signals. In this situation, the capture probe is used effectively, and the signal of one target is greater than that in 1:1 non-amplification strategy. More importantly, the strengthened signals in N:N amplification strategy are attributed to every participated target, while the signals in 1:1 non-amplification strategy are put down to one target. Therefore, the former has higher specificity.

Notably, such N:N strategy also works well in the artificial ion channels. The membrane ion channels are used by living organisms to communicate chemically and electronically with the extracellular world. In the closed state, they are impermeable to any ions and substances. Inspired by such genius designs of living systems, various artificial gatelike nanopores have been developed to imitate and to understand life process. Although substantial progress has been realized, the leak of ionic channel current in the closed state is still a challenge in fabricating efficient biomimetric nanopores. We successfully inserted the supersandwich structures into solid-state nanopores for the first time to construct highly efficient nanofluidic gating system [48]. The open-to-closed process was implemented by self-assembly of the supersandwich structures, and the closed-to-open process was realized by disassembly of the supersandwich structures through ATP-DNA binding interactions. We have demonstrated that this device exhibited high ON-OFF ratio (up to 10^6) and achieved the IMPLICATION logic operations in this system. More recently, we successfully constructed cross-linked DNA superstructures (3D) by using linker DNA

and Y-DNA units to successfully demonstrate highly efficient nanofluidic switch (Figure 4) [50]. The ON-OFF gating ratios were achieved 10^3-10^5 when DNA superstructured assembled in nanopores with diameters up to ca. 650 nm. The nanopores would be reopened via ATP-linker DNA interactions, the process of which can be accelerated by 20% ethanol. We demonstrated that the supersandwich performed well in the confined space such as nanopore.

More recently, our group also synthesized a bipolar probe consist of a hydrophilic DNA and a hydrophobic conjugated polymer (CP) [84]. In the aqueous solution, those bipolar probes would be aggregate and form micelles, quenching the fluorescence of CP. The aggregated bipolar probe was separated by adding telomerase, liberating fluorescence. N telomerases free N bipolar probes, therefore, it was a N:N amplification strategy. Such amplification strategy relies on the simultaneous participation of multiple signal probes and multiple targets, thus it is a group behavior, which benefits sensitivity and selectivity compared to 1:1 non-amplification strategy. On the basis of such properties of N:N amplification, we detected the 38 telomerase samples extracted from healthy or bladder cancer patients and demonstrated that the proposed method can successfully distinguish cancer patients from healthy persons. Different from the abovementioned principle, aggregation caused quenching (ACQ), we also developed an aggregation induced emission (AIE) method for telomerase detection [85]. We have demonstrated that this method had ability to detect telomerase in complex samples such as bloody urine samples and to distinguish the bladder cancer samples from healthy samples.

In comparison with 1:1 non-amplification strategy, the technology based on N:N amplification is a group behavior. It is more sensitive because multiple signal probes act as a whole in a reaction. It is more selectivity because the strong signal is attributed to every specific and accurate recognition of target.

3. Detection strategy based on the ratio of target to signal is 1:N

3.1 Enzymatic amplification based on DNA nanostructures

MB is an excellent example of a double-stranded nucleic acid (dsDNA) probe because it is specific, low cost, and easy to design and synthesize [3]. Although MB probe is suitable for detection in homogeneous solution and even in living cells, one target cause only one MB to emit fluorescence. The 1:1 stoichiometric ratio sets a limitation for the sensitive detection, and the detection limit can only be down to nanomolar range. In this context, various bioenzymes have been used to recycle the target to enhance sensitivity [19-42]. For example, Xie et al. reported a scheme to increase the detection sensitivity of MB by introducing nicking enzyme [36]. When the MB probe hybridized with target DNA, it was recognized and cleaved by nicking enzyme, releasing the target DNA, which gave rise to cleavage of hundreds of MBs and amplification of fluorescent signals (Figure 5). In such amplification strategy that depended on the nicking enzyme-assisted target recycling, the detection limit was greatly improved compared to that of 1:1 non-amplification strategy.

Whereas the signal amplification based on the nicking enzyme greatly enhance the sensitivity of DNA detection, challenges are still ahead of us. For example, nicking endonucleases are sequence-specific, there are many restrictions in the choice of

target sequences. To circumvent the above limitations, exonuclease III is widely used as an amplifying biocatalyst for biomarker detection. Exonuclease III selectively digests the blunt 3' terminus in duplex DNAs, giving a chance to selectively digest one of strands in duplex DNAs and free the other one. Plaxco et al. demonstrated a more versatile sequence-independent amplified DNA detection system by using MBs as signal probes and using exonuclease III to recycle target molecule [24]. Similarly, after hybridization with target, MB was opened, emitted fluorescence, exposed a blunt 3'terminus, and digested by exonuclease III, leading to the release of the target and starting anew cycle (Figure 6). This strategy had demonstrated a high magnitude of amplified, sensitive detection with a limit of 20 aM.

Besides exonuclease III, duplex-specific nuclease (DSN) is also utilized to overcome the drawbacks from nicking enzyme. For instance, Ye et al. created duplex specific nuclease signal amplification method to increase the sensitivity of sensors for microRNA (miRNA) [41]. In their method, taqman probe, a DNA linear probe with a fluorophore at its 5' terminus and a quencher at its 3' terminus, hybridized to the target miRNA and formed DNA:RNA duplex which can be recognized by DSN enzyme. The DSN enzyme digested DNA taqman probes and released target miRNA, thus one target molecule induced the cleavage of thousands of taqman probes, leading to a significant fluorescent signal amplification. They demonstrated their method allows the direct detection of miRNAs in the femtomolar range and showed a high selectivity for discriminating differences between miRNA family members.

DNA strand-displacement polymerase is another important type of bioenzyme

used to recycle the target. Wang et al. developed an amplified method for sensitive detection of DNA by employing strand-displacement polymerase and MB [23]. The target DNA can hybridize with the loop of the MB probe and open the stem. Then the primer annealed with the stem of the probe and triggered the polymerization reaction, in which process the target DNA was displaced and then hybridized to another probe, resulting in a circular polymerization reaction and amplified fluorescent signal for detection of trace amount of DNA. The proposed strategy achieved a high magnitude of amplification with a detection limit of 6.4×10^{-15} M.

3.2 Enzymatic amplification based on both DNA and non-DNA nanostructures

Advances in nanomaterials have benefited the development of isothermal amplifications. For instance, in order to achieve a detection with low-cost, high specificity, and rapid readout of a target, Liu et al. developed a colorimetric DNA detection technology by introducing a linker DNA complementary to target DNA, a DNA modified GNP probe, and a nicking endonuclease (NEase) (Figure 7) [37]. The linker DNA can assemble GNPs. If hybridized with the target DNA, the linker DNA would be recognized and nicked by NEase. After that the linker DNA would be dissociated from the target DNA. Subsequently, another intact linker DNA bound to the target and started a new recycle. The detection limit of 10 pM was realized. In order to overcome the limitation in the choice of target sequences from nicking enzyme, Yang and co-workers reported a universal platform by using DNA modified GNP probe, exonuclease III, and a linker DNA. They demonstrated a colorimetric

detection limit of 15 pM and single-base discrimination ability [86]. In such nicking enzyme/exonuclease III assisted colorimetric reaction, one target can trigger reactions many times and yield multiple signal outputs, we define such reaction as 1:N amplification. Additionally, our group developed a noninvasive, direct, and bidirectional method by taking advantage of difunctional GNP probes with multiple visual signals (Figure 8) [87]. One telomerase can initiate the extension reaction and produce an ssDNA containing multiple TTAGGG repeats [88-91]. After that, the repeats can hybridize with different reporter probes from different GNPs, forming complicated network structure. Because one telomerase brought multiple signal outputs, it was also a 1:N amplification. The GNP probes exhibited four states according to the concentration and activity of telomerase such as blue, purple, red, and precipitate, which was highly correlated with clinical diagnosis. 18 urine specimens from bladder cancer patients, inflammation patients, and healthy individuals were investigated to demonstrate the applicability for clinical diagnosis. One of the merits of the GNP-based assay is that the molecular recognition events can be observed by the naked eye or by UV-vis spectrometry.

Interestingly, Willner et al. firstly reported a multiplexed sensing platforms based on semiconductor QDs and exonuclease III (Figure 9) [32]. In comparison with any available organic fluorophore, QDs have superior photostability, broad adsorption, and narrow, size-tunable emission, which marks them as excellent potential donors. More importantly, different sized QDs can be excited simultaneously at a single wavelength. In their strategy, the nucleic acid modified at its 3’ end with the

black-hole quencher were linked to the QD, which led to the fluorescence quenching of QD through fluorescence resonance energy transfer (FRET). On hybridization with the target DNA, the nucleic acid would be digested by exonuclease III, activating the fluorescence of QD. They also successfully realized the multiplexed analysis of different target DNAs by using two different sized QDs. In this strategy, exonuclease recycled the target, emitting multiple fluorescent signals of MBs or QDs (1:N). The introduction of QDs makes the biosensor perform better in sensitivity and high throughput detection.

GO, another non-DNA nanostructure, prefers to interact with single-stranded DNA (ssDNA) over double-stranded DNA (dsDNA) because the exposed nucleobases in ssDNA have hydrophobic and p-stacking interactions with GO. Moreover, GO can not only protect DNA from nuclease because of the steric-hindrance effect but also quench the fluorescence of fluorophores conjugated with DNA due to the electronic transference. Based on those properties, Yang et al. developed an analyte-recycle assay by taking the advantage of GO and DNase I (Figure 10) [30]. GO can strongly bind aptamer and quench the fluorescence of fluorophores conjugated with aptamers. When challenged with a target, the aptamer bound to target and was released from GO. The free aptamer was captured and digested by DNase I, liberating fluorophore and releasing target. The released target triggered another cycle and therefore detection signals was enhanced. This 1:N amplification can achieve a detection limit of 50 nM for ATP. Furthermore, Tan and coworkers developed a Pb^{2+} sensor by taking the advantage of GR-5 DNAzyme and the remarkable differences

affinity between GO and ssDNA containing different number of bases in length [34]. They have demonstrated a detection limit of 300 pM for Pb^{2+} and a good selectivity when used in river water samples. The quenching ability of GO that is superior to the organic dyes and the specific surface area give opportunity to automatic detection.

3.3 Enzyme-free amplification based on DNA nanostructures

Although there is growing interest in constructing DNA supersandwich structure, the detection limit is still need to be improved because the ratio of target to signal probe is N:N. In our another study, we proposed an 1:N amplification strategy by employing an improved supersandwich structure which only contained one target DNA, greatly enhancing the detection limit. More interestingly, by integrating such improved structure within the nanopores, we developed a device that detects both subnanomolar DNA and sub-micromolar ATP (Figure 11) [44]. Analogous to supersandwich structure, hybridization chain reaction (HCR) was occurred when the initiator triggers a cascade hybridization of two stable species of DNA hairpins (Figure 12) [56]. In vivo, HCR was used to simultaneous mapping of multiple target mRNAs in situ [56-58]. In vitro, HCR was designed to isothermally detect ATP and nucleic acids without any enzyme [59-60]. More recently, nonenzymatic hairpin DNA cascade reaction have been attracted much interesting, in which DNA hybridization plays an important role [61-63]. Tan et al. developed a hairpin DNA cascade amplifier (HDCA) by introducing two hairpin structures to image miRNA expression in living cells. Target miRNA triggered the assembly of the hairpin H1 and H2 duplex, emitting fluorescence signal. Moreover, the target miRNA can be recycled and produced

multiple H1-H2 duplexes (Figure 13) [61]. In these strategy, one target yields multiple signals and they are 1:N amplification. Although the reaction based on the assembly of DNA requires longer time compared to that based on enzyme, it is more suitable for detection in living cells because it do not need transfect any commercial enzymes to living cells, simplifying the procedure.

3.4 Enzyme-free amplification based on both DNA and non-DNA nanostructures

Biobarcode firstly proposed by Mirkin and coworkers in 2003 is another kind of amplification method that can achieve polymerase chain reaction (PCR)-like sensitivity for both nucleic acid and protein without the help of enzymes [51-55]. The biobarcode strategy depends on two types of particles (Figure 14). One is the gold nanoparticle functionalized with multiple signal molecular modified oligonucleotides and a corresponding recognition agent. The other is magnetic microparticle (MMP) only modified with oligonucleotides partly complementary to the target or antibody. In the presence of the target, a sandwich complex, GNP-target-MMP, is formed. Thus, a target is corresponding to a large number of signal molecules, producing PCR-like sensitivity. Xing and coworkers developed an electrochemiluminescence method by using cysteamine as biobarcodes [51]. Their assay exhibited high selectivity for single-mismatched DNA in complex environment and achieved a detection limit as low as 100 fM. As we can see, one target captures one GNP, and one GNP attaches multiple signal molecules, therefore it is also 1:N amplification. In comparison with other 1:N amplification technologies dependent on bioenzymes, the biobarcode

assay is stable, cost-effective, and simple.

Besides GNP-based biobarcode technique, GNP-based colorimetry is another important method for enzyme-free detection [92]. For instance, our group reported a real-time colorimetric method based on DNA modified GNP probe to monitor the process of DNA strand displacement cascade. 14 DNA targets with different single-base mutations can be discriminated very well. Apart from those methods based on DNA-GNP conjugates, the enzyme-free amplification can be achieved using unmodified GNP by Sang and co-workers [93-94]. In their strategy, the hairpin auxiliary probes could prevent GNPs from salt-induced aggregation. However, the target DNA can induce cascade assembly of the auxiliary probe, in which process the branched junctions formed and target DNA was recycled. The branched junctions that consist of dsDNA could not stabilize GNPs against salt-induced aggregation, resulting in a red-to-blue color change.

In the 1:N amplification strategy, one target can consume multiple signal probes and emit strong signals, enormously intensifying the sensitivity. In comparison with 1:1 and N:N strategies, the technology based on 1:N amplification is more versatile and has more widely application. The highly sensitivity of 1:N amplification strategy makes for trace biomarkers detection in vivo.

4. Detection strategy based on the ratio of target to signal is 1:N^2

Isothermal oligonucleotides amplification based on 1:N amplification ratio is emerging as a potential amplification technique for rapid and cost-effective detection. However, advances in universality, sensitivity, and specificity for biomarker detection

are in continuous demand. Recently, exponential amplification have been realized by using improved MB or linear probe [19-22]. We developed a one-pot hairpin-mediated quadratic enzymatic amplification (HQEA) for miRNA detection by rational design of a functional MB (Figure 15) [20-21]. HQEA exhibited ultra-high sensitivity with a detection limit of 10 fM at 37 ℃ and 1 aM at 4 ℃ that corresponded to 9 strands of miRNA in a 15 μL sample, and showed high specificity with the ability to distinguish among miRNA family members. More importantly, HQEA had potential application in clinical research with great sensitivity and selectivity to crude extractions from MCF-7, PC3 cell lines, and even breast cancer tissues. Up to now, our group can design strategy with 1:1, 1:N, 1:N^2 reaction ratio via controlling the number and types of bioenzymes. Cheng et al. demonstrated another exponential miRNA assay by introducing an amplification template containing two repeat sequences that were complementary to the target miRNA [22]. They have demonstrated that the linear probe, polymerase, nicking enzyme assisted amplification method can detect miRNAs as low as 0.1 zmol, and the dynamic range was more than 10 orders of magnitude. In the above mentioned both two strategies, one target can trigger two linear amplification simultaneously, thus they are both 1:N^2 amplification.

Meanwhile, various exponential-amplification methods have been developed by employing both DNA and non-DNA nanostructures [95-98]. For example, Tang et al. developed a miRNA assay with sensitivity and specificity based on the enzyme assisted cooperative amplification and fluorescence quenching ability of GO [95].

Zhang et al. also reported an isothermal exponential amplification reaction (EXPAR) for colorimetric detection of transcription factors, NF-κB p50 [97]. In the presence of NF-κB p50, the GNP experienced a red-to-purple color change after the processes of protein-DNA interactions, exonuclease III digestion, and isothermal exponential amplification. They have demonstrated a detection limit of 3.8 pM for NF-κB p50, which was improved by as much as 4 orders of magnitude compared to the conventional GNP-based colorimetric assay. More recently, Zhang et al. reported an exponential amplification assay for miRNA detection by employing miRNA-triggered two-stage amplification and QDs [98]. The products from the amplification reaction can hybridize with capture and Cy5-labeled reporter probes to form sandwich structures which can be assembled on the surface of 605QDs. When excited by a wavelength of 488 nm, the fluorescent signal of Cy5 can be detected due to FRET from 605QD to Cy5. Although the sensitivity of exponential amplification is attractive, the non-specific reaction is also increased. The involvement of non-DNA nanostructures is beneficial to ensure the accuracy of biosensor.

5. Conclusion and Perspective

In this article, we have highlighted several examples to summarize the drawbacks and improvements occurred in the development of isothermal amplification. We also show how the high efficient amplification can be achieved by using a variety of DNA and non-DNA nanostructures with different dimensions such as sandwich structure, DNA superstructure, QD, biobarcode probe, GO, GNP,

improved MB and DNA linear probe. Among them, DNA nanostructures have strong programmable ability, which can be used to construct multiplexed biosensor in complicated environment. Non-DNA nanostructures have a high specific surface area and attractive mechanical, electronic, and optical properties, which makes for the miniaturization and diversity of biosensor.

However, to improve analysis method in selectivity, high speed, specificity, accuracy, and automation is also a challenge ahead of us and is still in demand. In vitro, we expect that the method with $1:N^n$ amplification ratio for biomarkers detection can be realized by using multidisciplinary sciences. Meanwhile, we also happy to see more smart nanostructures can be designed and coupled to conventional analytical techniques to construct excellent biosensors with simple detection procedures.

On the other hand, Pierce et al. employed the HCR to image the multiplex mRNA in fixed cells and tissues [56-57]. Tan et al. developed a non-enzymatic hairpin DNA cascade reaction to image specific mRNA in living cells [61]. Although there are great achievements in isothermal amplification in vivo, many efforts still should be made in enhancing selectivity and decreasing background noise. Furthermore, the efficiency of cellular delivery of nucleic acid probes is also a key factor in vivo detection. Meanwhile, increasing the stability of probes in enzyme resistance can help researchers to understand the life process, and then to diagnose and treat cancer. With the development of biology and nanoscience, we hope more intelligent isothermal amplification strategies with simple operation can be developed and

applied to track the biomarkers in vivo.

Acknowledgements

This research is supported by National Basic Research Program of China (973 program, 2015CB932600, 2013CB933000), National Natural Science Foundation of China (21525523, 21375042, 21574048, 21405054), 1000 Young Talent (to Fan Xia) and initiatory financial support from HUST.

References

1. E. Farjami, R. Campos, J. Nielsen, K. Gothelf, J. Kjems, E. E. Ferapontova, *Anal. Chem.* 2013, 85, 121.
2. A. Idili, A. Amodio, M. Vidonis, J. Feinberg-Somerson, M. Castronovo, F. Ricci, *Anal. Chem.* 2014, 86, 9013.
3. S. Tyagi, F. R. Kramer, *Nat. Biotechnol*. 1996, 14, 303.
4. B. Dubertret, M. Calame, A. J. Libchaber, *Nat. Biotechnol.* 2001, 19, 365.
5. F. Xia, X. Zuo, R. Yang, Y. Xiao, D. Kang, A. Vallée-Bélisle, X. Gong, A. J. Heeger, K. W. Plaxco, *J. Am. Chem. Soc.* 2010, 132, 1252.
6. R. Qian, L. Ding, L. Yan, M. Lin, H. Ju, *J. Am. Chem. Soc.* 2014, 136, 8205.
7. P. Conlon, C. J. Yang, Y. Wu, Y. Chen, K. Martinez, Y. Kim, N. Stevens, A. A. Marti, S. Jockusch, N. J. T urro, W. Tan, *J. Am. Chem. Soc.* 2008, 130, 336.
8. S. Song, Z. Liang, J. Zhang, L. Wang, G. Li, C. Fan, *Angew. Chem. Int. Ed.* 2009, 48, 8670.
9. W. Pan, T. Zhang, H. Yang, W. Diao, N. Li, B. Tang, *Anal. Chem.* 2013, 85, 10581.
10. K. Wang, Z. Tang, C. J. Yang, Y. Kim, X. Fang, W. Li, Y. Wu, C. D. Medley, Z. Cao, J. Li, P. Colon, H. Lin, W. Tan, *Angew. Chem. Int. Ed.* 2009, 48, 856.
11. E. Farjami, L. Clima, K. V. Gothelf, E. E. Ferapontova, *Anal. Chem.* 2011, 83, 1594.
12. X. Zuo, Y. Xiao, K. W. Plaxco, *J. Am. Chem. Soc.* 2009, 131, 6944.
13. Z. Lin, G. Zhang, W. Yang, B. Qiu, G. Chen. *Chem. Commun.* 2012, 48, 9918.
14. Y. Xiang, Y. Lu, *Anal. Chem.* 2012, 84, 1975.
15. J. Shen, Y. Li, H. Gu, F. Xia, X. Zuo, *Chem. Rev.* 2014, 114, 7631.

16. M. Lin, J. Wang, G. Zhou, J. Wang, N. Wu, J. Lu, J. Gao, X. Chen, J. Shi, X. Zuo, C. Fan, *Angew. Chem. Int. Ed.* 2015, 54*,* 2151.

17. H. Pei, X. Zuo, D. Zhu, Q. Huang, C. Fan, *Acc. Chem. Res.* 2014, 47, 550.

18. H. Pei, N. Lu, Y. Wen, S. Song, Y. Liu, H. Yan, C. Fan, *Adv. Mater.* 2010, 22, 4754.

19. A. R. Connolly, M. Trau, *Angew. Chem. Int. Ed.* 2010, 49, 2720.

20. R. Duan, X. Zuo, S. Wang, X. Quan, D. Chen, Z. Chen, L. Jiang, C. Fan, F. Xia, *J. Am. Chem. Soc.* 2013, 135, 4604.

21. R. Duan, X. Zuo, S. Wang, X. Quan, D. Chen, Z. Chen, L. Jiang, C. Fan, F. Xia, *Nat. Protoc.* 2014, 9, 597.

22. H. Jia, Z. Li, C. Liu, Y. Cheng, *Angew. Chem. Int. Ed.* 2010, 49, 5498.

23. Q. Guo, X. Yang, K. Wang, W. Tan, W. Li, H. Tang, H. Li, *Nucleic Acids Res*. 2009, 37, e20.

24. X. Zuo, F. Xia, Y. Xiao, K. W. Plaxco, *J. Am. Chem. Soc.* 2010, 132, 1816.

25. E. Ju, X. Yang, Y. Lin, F. Pu, J. Ren, X. Qu, *Chem. Commun*. 2012, 48, 11662.

26. J. Su, H. Zhang, B. Jiang, H. Zheng, Y. Chai, R. Yuan, Y. Xiang, *Biosens. Bioelectron.* 2011, 29, 184.

27. L. Cui, Z. Chen, Z. Zhu, X. Lin, X. Chen, C. J. Yang, *Anal. Chem.* 2013, 85, 2269.

28. F. Xuan, X. Luo, I. M. Hsing, *Anal. Chem.* 2013, 85, 4586.

29. L. Peng, Z. Zhu, Y. Chen, D. Han, W. Tan, *Biosens. Bioelectron.* 2012, 35, 475.

30. C. H. Lu, J. Li, M. H. Lin, Y. W. Wang, H. H. Yang, X. Chen, G. N. Chen, *Angew. Chem. Int. Ed.* 2010, 49, 8454.

31. S. Wu, P. Liang, H. Yu, X. Xu, Y. Liu, X. Lou, Y. Xiao, *Anal. Chem.* 2014, 86, 3461.

32. R. Freeman, X. Liu, I. Willner, *Nano Lett.* 2011, 11, 4456.

33. R. M. Kong, X. B. Zhang, L. L. Zhang, Y. Huang, D. Q. Lu, W. Tan, G. L. Shen, R. Q. Yu, *Anal. Chem.* 2011, 83, 14.

34. X. H. Zhao, R. M. Kong, X. B. Zhang, H. M. Meng, W. N. Liu, W. Tan, G. L. Shen, R. Q. Yu, *Anal. Chem*. 2011, 83, 5062.

35. J. Li, H. E. Fu, L. J. Wu, A. X. Zheng, G. N. Chen, H. H. Yang, *Anal. Chem.* 2012, 84, 5309.

36. J. J. Li, Y. Chu, B. Y. Lee, X. S. Xie, *Nucleic Acids Res.* 2008, 36, e36.

37. W. Xu, X. Xue, T. Li, H. Zeng, X. Liu, *Angew. Chem. Int. Ed.* 2009, 48, 6849.

38. W. Shen, H. Deng, Y. Ren, Z. Gao, *Chem. Commun.* 2013, 49, 4959.

39. F. Degliangeli, P. Kshirsagar, V. Brunetti, P. P. Pompa, R. Fiammengo, *J. Am. Chem. Soc.* 2014, 136, 2264.

40. X. Lin, C. Zhang, Y. Huang, Z. Zhu, X. Chen, C. J. Yang, *Chem. Commun.* 2013, 49, 7243.

41. B. C. Yin, Y. Q. Liu, B. C. Ye, *J. Am. Chem. Soc.* 2012, 134, 5064.

42. Y. V. Gerasimova, D. M. Kolpashchikov, *Chem. Soc. Rev.* 2014, 43, 6405.

43. X. Chen, Y. H. Lin, J. Li, L. S. Lin, G. N. Chen, H. H. Yang, *Chem. Commun*. 2011, 47, 12116.

44. N. Liu, Y. Jiang, Y. Zhou, F. Xia, W. Guo, L. Jiang, *Angew. Chem. Int. Ed.* 2013, 52, 2007.

45. F. Xia, R. J. White, X. Zuo, A. Patterson, Y. Xiao, D. Kang, X. Gong, K. W. Plaxco, A. J. Heeger, *J. Am. Chem. Soc.* 2010, 132, 14346.

46. C. Li, Z. Wang, T. Gao, A. Duan, G. Li, *Chem. Commun*. 2013, 49, 3760.

47. T. Yuan, Z. Liu, L. Hu, L. Zhang, G. Xu, *Chem. Commun.* 2011, 47, 11951.

48. Y. Jiang, N. Liu, W. Guo, F. Xia, L. Jiang, *J. Am. Chem. Soc.* 2012, 134, 15395.

49. W. Song, C. Lau, J. Lu, *Analyst*. 2012, 137, 1611.

50. W. Guo, F. Hong, N. Liu, J. Huang, B. Wang, R. Duan, X. Lou, F. Xia, *Adv. Mater.* 2015, 27, 2090.

51. R. Duan, X. Zhou, D. Xing, *Anal. Chem.* 2010, 82, 3099.

52. X. Zhou, R. Duan, D. Xing, *Analyst*. 2012, 137, 1963.

53. J. M. Nam, S. I. Stoeva, C. A. Mirkin, *J. Am. Chem. Soc.* 2004, 126, 5932.

54. J. M. Nam, C. S. Thaxton, C. A. Mirkin, *Science* 2003, 301, 1884.

55. D. Zhu, Y. Tang, D. Xing, W. R. Chen. *Anal. Chem*. 2008, 80, 3566.

56. H. M. Choi, V. A. Beck, N. A. Pierce, *ACS Nano* 2014, 8, 4284.

57. H. M. Choi, J. Y. Chang, A. Trinhle, J. E. Padilla, S. E. Fraser, N. A. Pierce, *Nat. Biotechnol.* 2010, 28, 1208.

58. Z. Wu, G. Q. Liu, X. L. Yang, J. H. Jiang, *J. Am. Chem. Soc.* 2015, 137, 6829.

59. F. Wang, J. Elbaz, R. Orbach, N. Magen, I. Willner, *J. Am. Chem. Soc.* 2011, 133, 17149.

60. R. M. Dirks, N. A. Pierce, *Proc. Natl. Acad. Sci. USA* 2004, 101, 15275.

61. C. Wu, S. Cansiz, L. Zhang, I. T. Teng, L. Qiu, J. Li, Y. Liu, C. Zhou, R. Hu, T. Zhang, C. Cui, L. Cui, W. Tan, *J. Am. Chem. Soc.* 2015, 22, 4900.

62. B. Li, Y. Jiang, X. Chen, A. D. Ellington. *J. Am. Chem. Soc.* 2012, 134, 13918.

63. X. Chen, N. Briggs, J. R. McLain, A. D. Ellington, *Proc. Natl. Acad. Sci. USA* 2013,

110, 5386.

64. M. Swierczewska, G. Liu, S. Lee, X. Chen, *Chem. Soc. Rev.* 2012, 41, 2641.
65. J. J. Xu, W. W. Zhao, S. Song, C. Fan, H. Y. Chen. *Chem. Soc. Rev.* 2014, 43, 1601.
66. M. Suzuki, Y. Husimi, H. Komatsu, K. Suzuki, K. T. Douglas, *J. Am. Chem. Soc.* 2008, 130, 5720.
67. C. Y. Zhang, J. Hu, *Anal. Chem.* 2010, 82, 1921.
68. X. Xie, W. Xu, X. Liu, *Acc. Chem. Res.* 2012, 45, 1511.
69. M. C. Daniel, D. Astruc, *Chem. Rev.* 2004, 104, 293.
70. P. D. Howes, R. Chandrawati, M. M. Stevens, Science 2014, 346, 1247390.
71. K. Saha, S. S. Agasti, C. Kim, X. Li, V. M. Rotello, *Chem. Rev.* 2012, 112, 2739.
72. C. Chung, Y. K. Kim, D. Shin, S. R. Ryoo, B. H. Hong, D. H. Min, *Acc. Chem. Res.* 2013, 46, 2211.
73. D. Chen, H, Feng, J. Li, *Chem. Rev.* 2012, 112, 6027.
74. K. S. Novoselov, A. K. Geim, S. V. Morozov, D. Jiang, Y. Zhang, S. V. Dubonos, I. V. Grigorieva, A. A. Firsov, *Science* 2004, 306, 666.
75. A. K. Geim, *Science* 2009, 324, 1530.
76. G. Eda, G. Fanchini, M. Chhowalla, *Nat. Nanotechnol.* 2008, 3, 270.
77. W. W. Cai, Y. W. Zhu, X. S. Li, R. D. Piner, R. S. Ruoff, *Appl. Phys. Lett.* 2009, 95, 123115.
78. X. Wang, L. J. Zhi, K. Mullen, *Nano Lett*. 2008, 8, 323.
79. H. A. Becerril, J. Mao, Z. Liu, R. M. Stoltenberg, Z. Bao, Y. Chen, *ACS Nano* 2008, 2, 463.

80. Y. Wang, L. Tang, Z. Li, Y. Lin, J. Li, *Nat. Protoc.* 2014, 9, 1944.

81. H. Wang, H. Feng, J. Li, *Small* 2014, 10, 2165.

82. L. Tang, D. Li, J. Li, *Chem. Commun*. 2013, 49, 9971.

83. L. Tang, Y. Wang, Y. Liu, J. Li, *ACS Nano*. 2011, 5, 3817.

84. Y. Jia, X. Zuo, Lou X, M. Miao, Y. Cheng, X. Min, X. Li, F. Xia, *Anal. Chem*. 2015, 87, 3890.

85. X. Lou, Y. Zhuang, X. Zuo, Y. Jia, Y. Hong, X. Min, Z. Zhang, X. Xu, N. Liu, F. Xia, B. Z. Tang, *Anal. Chem*. 2015, 87, 6822.

86. L. Cui, G. Ke, W. Y. Zhang, C. J. Yang, *Biosens. Bioelectron.* 2011, 26, 2796.

87. R. Duan, B. Wang, T. Zhang, Z. Zhang, S. Xu, Z. Chen, X. Lou, F. Xia, *Anal. Chem.* 2014, 86, 9781.

88. J. Wang, L, Wu, J. Ren, X. Qu, *Small* 2012, 8, 259.

89. J. Wang, X. Qu, *Nanoscale* 2013, 5, 3589.

90. J. Wang, L. Wu, J. Ren, X. Qu, *Nanoscale* 2014, 6, 1661.

91. L. Wu, J. Wang, J. Ren, X. Qu, *Adv. Funct. Mater.* 2014, 24, 2727.

92. R. Duan, B. Wang, F. Hong, T. Zhang, Y. Jia, J. Huang, A. Hakeem, N. Liu, X. Lou, F. Xia, *Nanoscale*. 2015, 7, 5719.

93. W. Yun, J. Jiang, D. Cai, P. Zhao, J. Liao, G. Sang, *Biosens. Bioelectron.* 2015, 77, 421.

94. P. Liu, X. Yang, S. Sun, Q. Wang, K. Wang, J. Huang, J. Liu, L. He, *Anal. Chem.* 2013, 85, 7689.

95. H. Liu, L. Li, Q. Wang, L. Duan, B. Tang, *Anal. Chem*. 2014, 86, 5487.

96. Y. Zhang, C. Y. Zhang, *Anal. Chem*. 2012, 84, 224.

97. Y. Liao, X. Zhou, D. Xing, *ACS Appl. Mater. Interfaces* 2014, 6, 9988.

98. Y. Zhang, J. Hu, C. Y. Zhang, *Anal. Chem*. 2012, 84, 9544.

Figure captions

Figure 1. Schematic illustration of nanostructure assisted isothermal amplification in biosensors

Figure 2. A, Structure and function of MB. Reproduced with permission.[10] 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. B, Multiplexed detection using MB modified GNP. Reproduced with permission.[8] 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. C, DNA detection based on the sandwich structure. Reproduced with permission.[15] 2014 American Chemical Society. D, The construction of TSP and its application in nucleic acid detection. Reproduced with permission.[18] 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 3. Schematic representation of a traditional sandwich assay and a supersandwhich assay. Reproduced with permission.[45] 2010 American Chemical Society.

Figure 4. Build 3D superstructures in solid-state nanopores to act as efficient gatekeepers. Reproduced with permission.[50] 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 5. The 1:N isothermal amplification for DNA detection based on MB and nicking enzyme. reproduced with permission.[36] 2008 Oxford University Press.

Figure 6. The 1:N isothermal amplification for DNA detection based on MB and exonuclease III. reproduced with permission.[24] 2010 American Chemical Society.

Figure 7. The 1:N isothermal amplification for DNA detection based on GNPs. reproduced with permission.[37] 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 8. The 1:N isothermal amplification for telomerase detection based on GNPs. reproduced with permission.[87] 2014 American Chemical Society.

Figure 9. The 1:N isothermal amplification for DNA detection based on QDs. reproduced with permission.[32] 2010 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 10. The 1:N isothermal amplification for DNA detection based on GO. reproduced with permission.[30] 2011 American Chemical Society.

Figure 11. Nanopore sensors based on supersandwich structure for ATP and DNA detection. Reproduced with permission.[44] 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 12. Hybridization chain reaction, a programmable molecular amplifier, for mRNA detection in zebrafish embryos. Reproduced with permission.[56] 2014 American Chemical Society.

Figure 13. Hairpin DNA cascade amplifier for mRNA detection in living cells. Reproduced with permission.[61] 2015 American Chemical Society.

Figure 14. Detection of prostate-specific antigen based on biobarcode method. Reproduced with permission.[54] 2003 American Association for the Advancement of Science.

Figure 15. Schematic illustration of $1:N^2$ amplification ratio for miRNA detection. Reproduced with permission.[20] 2013 American Chemical Society. B, The structure of improved MB. Reproduced with permission.[21] 2014 Nature Publishing Group.

Figure 1

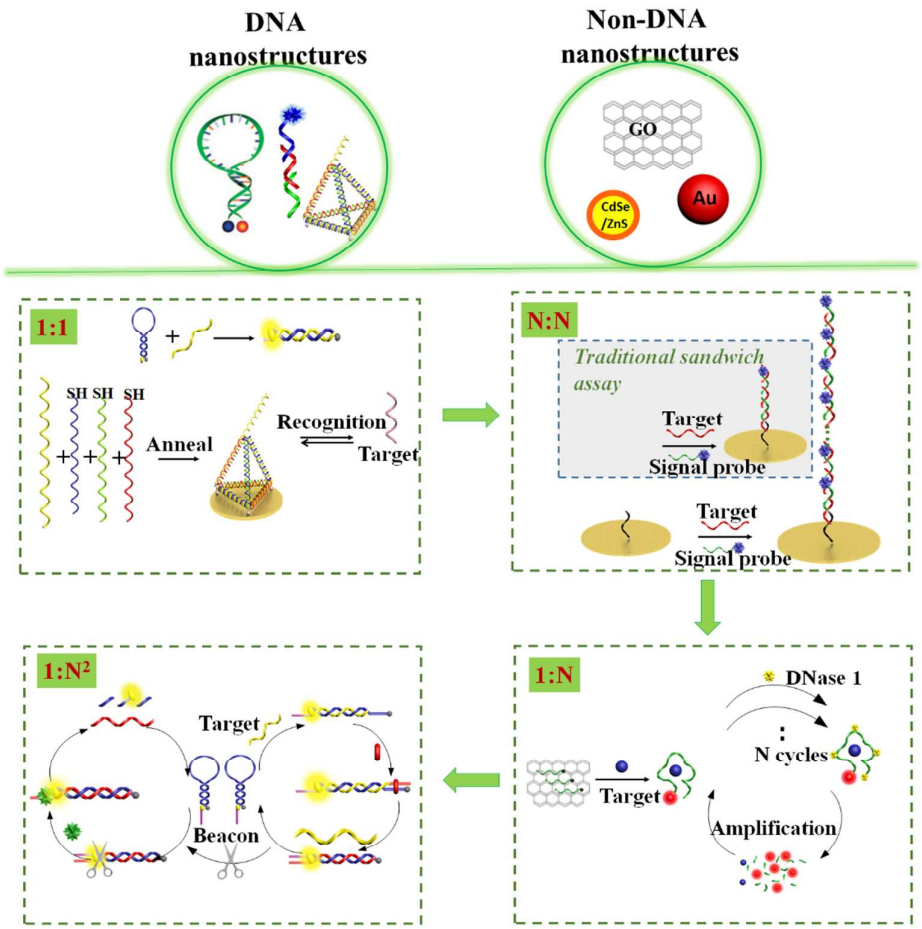

DNA nanostructures
Non-DNA nanostructures
GO
CdSe /ZnS
Au
1:1
SH SH SH
Anneal
Recognition
Target
N:N
Traditional sandwich assay
Target
Signal probe
Target
Signal probe
1:N
DNase 1
N cycles
Target
Amplification
1:N²
Target
Beacon

Figure 2

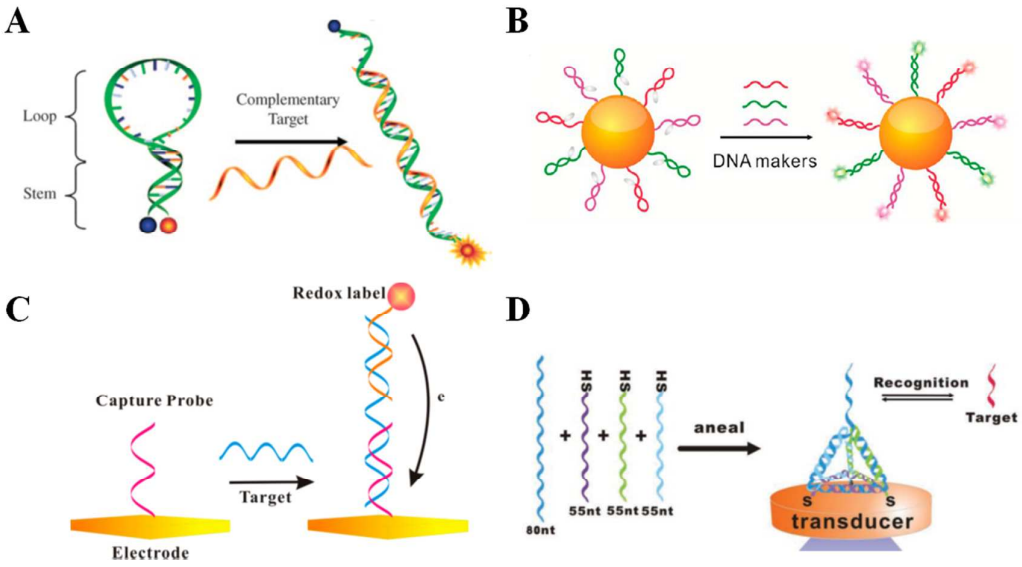

A
Loop
Stem
Complementary Target
B
DNA makers
C
Redox label
e
Capture Probe
Target
Electrode
D
HS
HS
HS
55nt 55nt 55nt
80nt
aneal
Recognition
Target
S
S
transducer

Figure 3

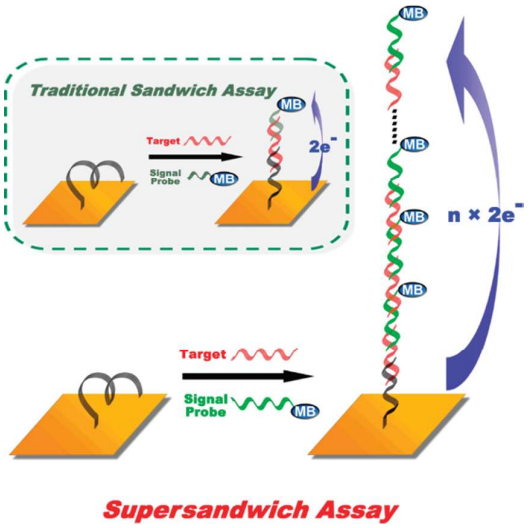

Traditional Sandwich Assay
Target
Signal Probe
MB
2e⁻
n × 2e⁻
Supersandwich Assay

Figure 4

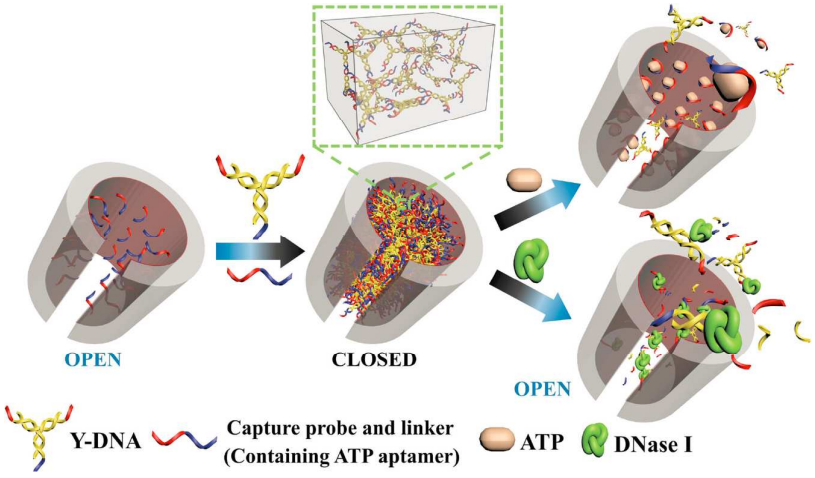

OPEN
CLOSED
OPEN
Y-DNA
Capture probe and linker
(Containing ATP aptamer)
ATP
DNase I

Figure 5

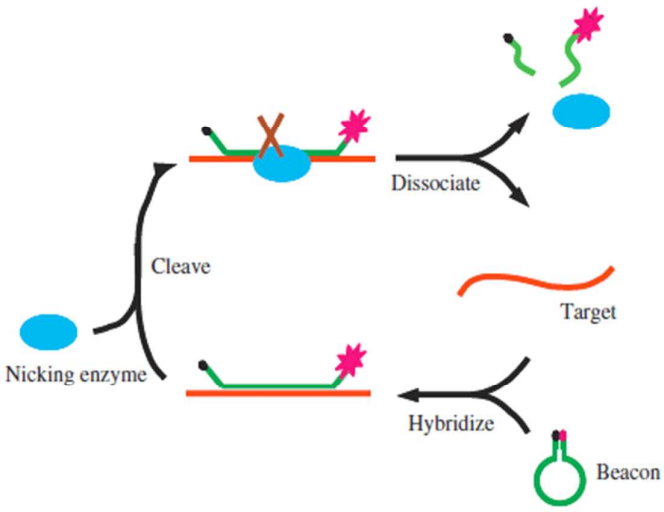

Dissociate
Cleave
Target
Nicking enzyme
Hybridize
Beacon

Figure 6

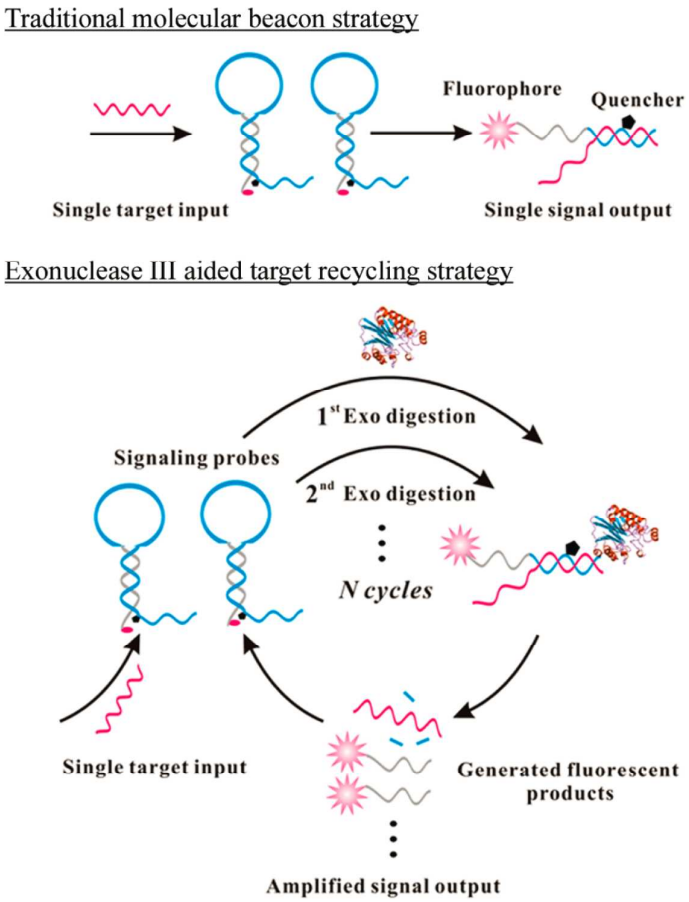

Traditional molecular beacon strategy
Fluorophore
Quencher
Single target input
Single signal output
Exonuclease III aided target recycling strategy
1st Exo digestion
Signaling probes
2nd Exo digestion
N cycles
Single target input
Generated fluorescent products
Amplified signal output

Figure 7

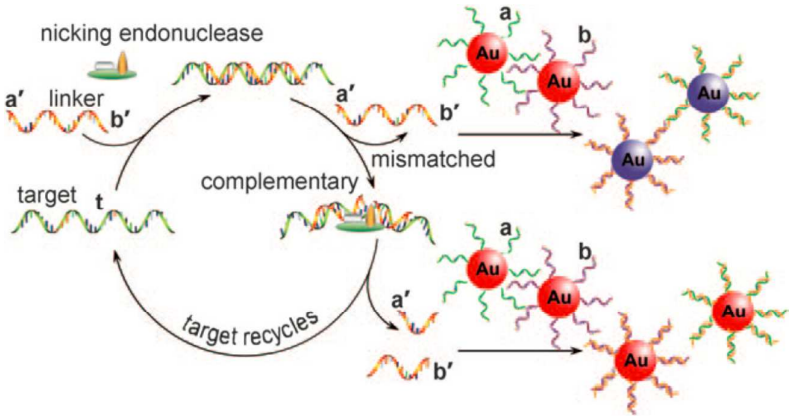

nicking endonuclease
a′
linker
b′
a′
b′
mismatched
target
t
complementary
target recycles
a′
b′
a
b
Au

Figure 8

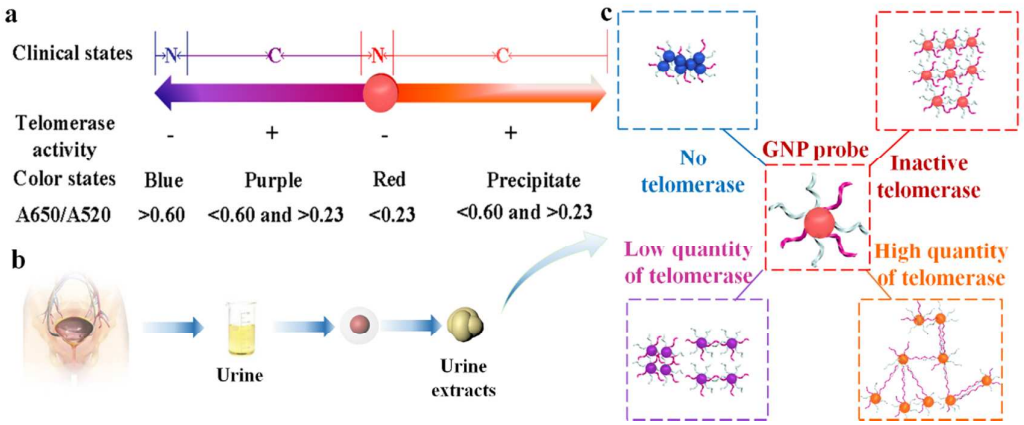

a
Clinical states
N
C
N
C
Telomerase activity
-
+
-
+
Color states
Blue
Purple
Red
Precipitate
A650/A520
>0.60
<0.60 and >0.23
<0.23
<0.60 and >0.23
b
Urine
Urine extracts
c
No telomerase
GNP probe
Inactive telomerase
Low quantity of telomerase
High quantity of telomerase

Figure 9

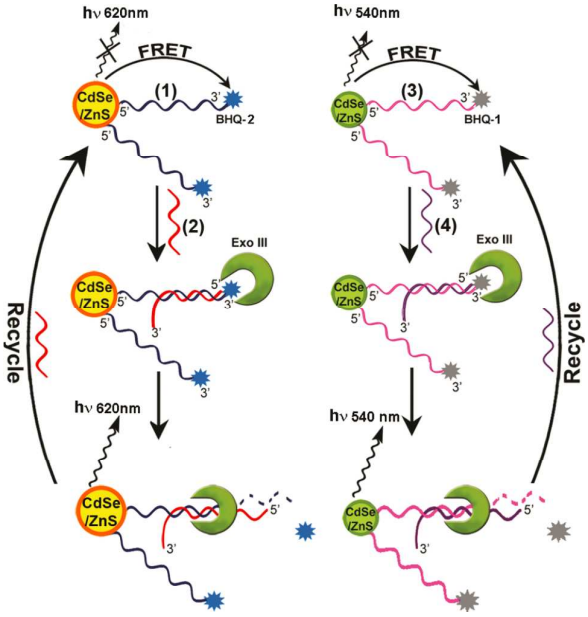

hν 620nm
FRET
(1)
CdSe /ZnS
BHQ-2
(2)
Exo III
Recycle
hν 620nm
hν 540nm
FRET
(3)
BHQ-1
(4)
Exo III
hν 540 nm
Recycle

Figure 10

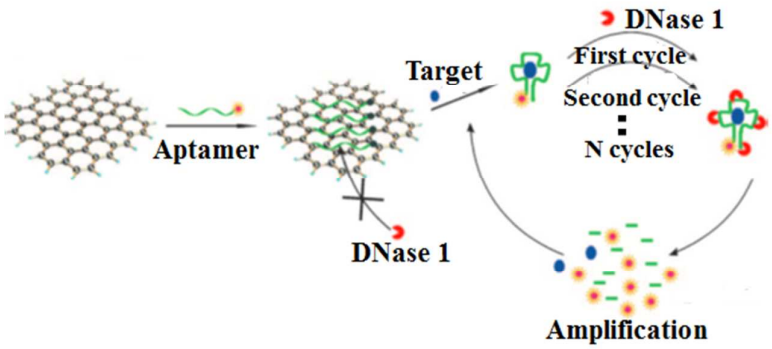

DNase 1
First cycle
Second cycle
N cycles
Target
Aptamer
DNase 1
Amplification

Figure 11

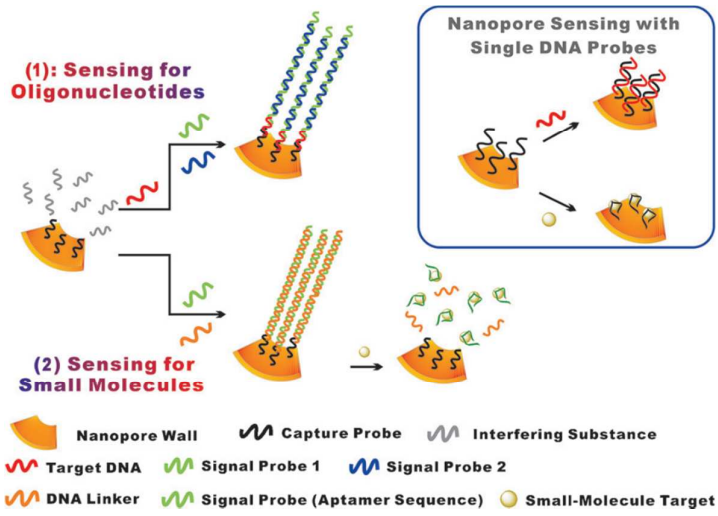

Nanopore Sensing with Single DNA Probes
(1): Sensing for Oligonucleotides
(2) Sensing for Small Molecules
Nanopore Wall
Capture Probe
Interfering Substance
Target DNA
Signal Probe 1
Signal Probe 2
DNA Linker
Signal Probe (Aptamer Sequence)
Small-Molecule Target

Figure 12

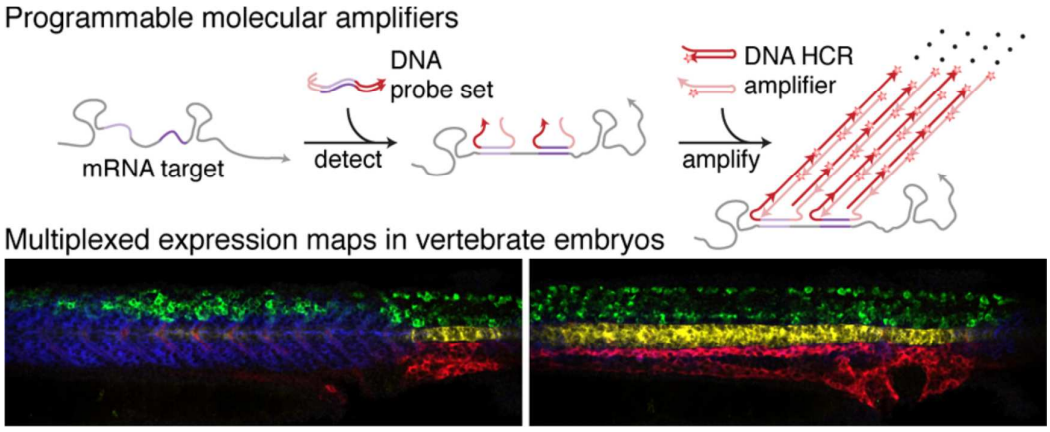

Programmable molecular amplifiers
DNA
probe set
DNA HCR
amplifier
mRNA target
detect
amplify
Multiplexed expression maps in vertebrate embryos

Figure 13

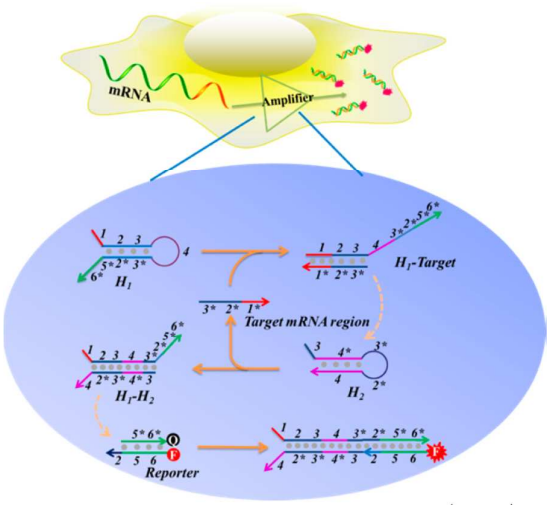

mRNA
Amplifier
H1
H1-Target
Target mRNA region
H2
H1-H2
Reporter

Figure 14

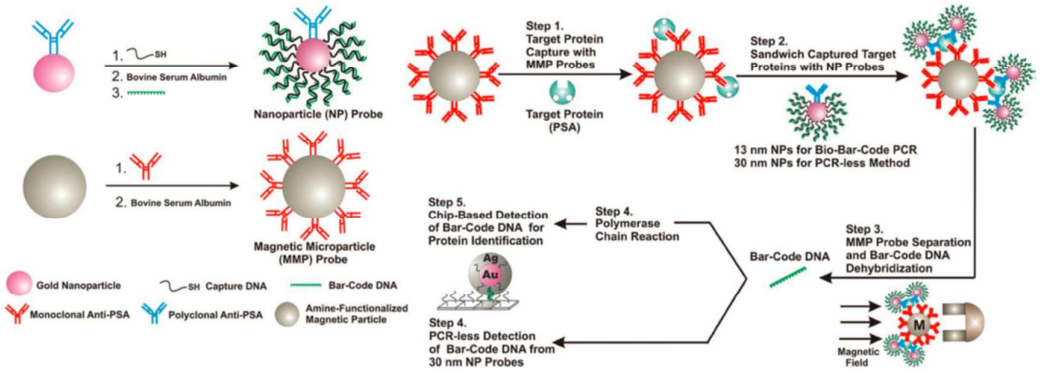
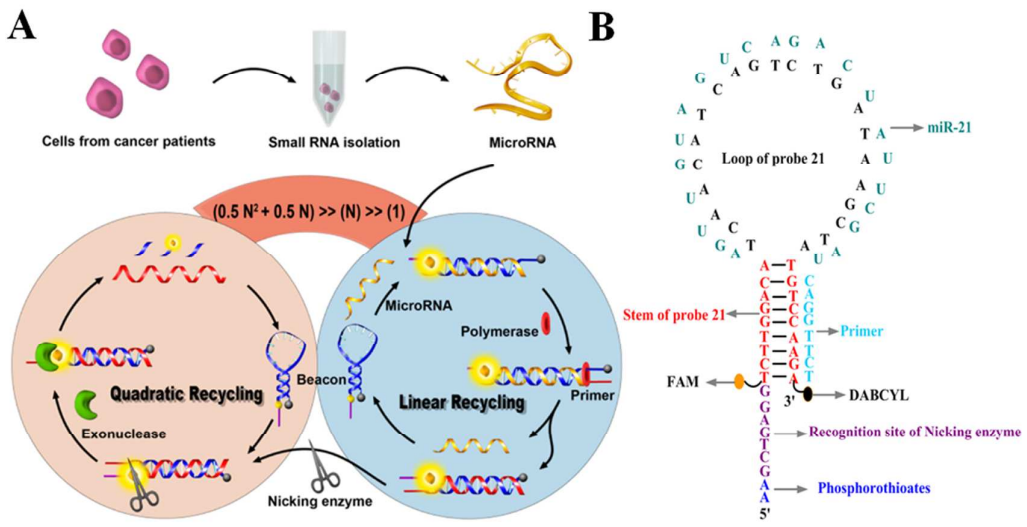

1. SH
2. Bovine Serum Albumin
3.
Nanoparticle (NP) Probe
1.
2. Bovine Serum Albumin
Magnetic Microparticle (MMP) Probe
Gold Nanoparticle
SH Capture DNA
Bar-Code DNA
Monoclonal Anti-PSA
Polyclonal Anti-PSA
Amine-Functionalized Magnetic Particle
Step 1. Target Protein Capture with MMP Probes
Target Protein (PSA)
Step 2. Sandwich Captured Target Proteins with NP Probes
13 nm NPs for Bio-Bar-Code PCR
30 nm NPs for PCR-less Method
Step 3. MMP Probe Separation and Bar-Code DNA Dehybridization
Bar-Code DNA
Magnetic Field
Step 4. Polymerase Chain Reaction
Step 5. Chip-Based Detection of Bar-Code DNA for Protein Identification
Ag
Au
Step 4. PCR-less Detection of Bar-Code DNA from 30 nm NP Probes

Figure 15


A
Cells from cancer patients
Small RNA isolation
MicroRNA
(0.5 N² + 0.5 N) >> (N) >> (1)
MicroRNA
Polymerase
Beacon
Quadratic Recycling
Linear Recycling
Primer
Exonuclease
Nicking enzyme
B
Loop of probe 21
miR-21
Stem of probe 21
Primer
FAM
3'
DABCYL
Recognition site of Nicking enzyme
Phosphorothioates
5'