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INVITED REVIEW

DNA Technology-assisted Signal Amplification Strategies in Electrochemiluminescence Bioanalysis

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Abstract
Sensitive and accurate detection of biological analytes, such as proteins, genes, small molecules, ions, cells, etc., has been a significant project in life science. Signal amplification is one of the most effective approaches to improve the sensitivity of bioanalysis. Taking advantage of specific base pairing, programmable operation, and predictable assembly, DNA is flexible and suitable to perform the signal amplification procedure. In recent years, signal amplification strategies by means of DNA technology have been widely integrated into the construction of electrochemiluminescence (ECL) biosensors, achieving desirable analytical performance in clinical diagnosis, biomedical research, and drug development. To the best of our knowledge, these DNA signal amplification technologies mainly include classical polymerase chain reaction, and various amplification approaches conducted under mild conditions, such as rolling circle amplification (RCA) or hyperbranched RCA, cleaving enzyme-assisted amplification, DNAzyme-involved amplification, toehold-mediated DNA strand displacement amplification without enzyme participation, and so on. This review overviews the recent advancements of DNA signal amplification strategies for bioanalysis in the ECL realm, sketching the creative trajectory from strategies design to ultrasensitive ECL platform construction and resulting applications.

Keywords Electrochemiluminescence · DNA technology · Signal amplification strategies · Bioanalysis

1 Introduction

Since the first detailed studies on electrochemiluminescence (ECL) originated from the papers published by Hercules [1] and Bard [2] in the 1960s, ECL has been acknowledged as a versatile analytical technique and widely exploited in numerous applications, especially bioanalysis [3–5]. ECL describes a photoemission phenomenon that occurs during the energy relaxation of excited substances triggered by a modulated potential in the vicinity of the electrode [6, 7]. ECL makes a combination of electrochemistry (EC) and chemiluminescence (CL) with both advantages, such as remarkable sensitivity, wide dynamic range, cheap instruments, and simple operations [8–10]. Moreover, ECL shows inherent superiorities of easy regulation by the applied potential and almost no background noise due to the absence of external excitation light sources [11, 12]. The dominant ECL mechanisms are usually divided into two categories: annihilation and coreactant mechanism. The former transmits an ECL signal electrogenerated from single emitters, and the latter usually requires a suitable coreactant assisting emitters to output the ECL intensity [9]. In the ECL realm, developing advanced strategies for highly sensitive bioanalysis is in urgent demand to meet the requirements of clinical diagnosis, biomedical research, and pharmaceutical development [13, 14].

Signal amplification is one of the most effective approaches to improve the sensitivity of bioanalysis [5]. General signal amplification strategies sprang up in the past decades, covering the design of novel efficient ECL system with powerful luminophores, suitable coreactants, efficient redox probes, functional nanomaterial matrixes, and the proposal of skillful analysis strategies. Taking advantage of specific base pairing, programmable operation, and predictable assembly, DNA is flexible and suitable to perform the signal amplification procedure. To the best of our knowledge, DNA amplification technologies used in the ECL domain mainly include classical polymerase chain reaction (PCR), various

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mild amplification approaches, such as rolling circle amplification (RCA) or hyperbranched RCA (HRCA), endonuclease or exonuclease-assisted amplification, DNAzyme-involved amplification, toehold-mediated nonenzymic strand displacement amplification (TSDA), and so on. These versatile amplification strategies have been widely integrated into the manufacture of ECL biosensors to achieve higher analytical performance.

This review overviews the recent ECL bioanalysis strategies with a more detailed emphasis on the advanced DNA signal amplification technologies. Several representative ones are summarized in Table 1, and others will be described in more detail below. Nevertheless, there have been hundreds of published papers covering this field in recent years, and it is a pity that we are incapable of referring to them wholly. We apologize to those authors whose researches are not mentioned here. At last, this work outlines brief future trends and perspectives of the DNA technology-based signal amplification strategies in ECL bioanalysis.

2 Polymerase-assisted Amplifications in ECL Bioanalysis

Polymerases are a class of enzymes that specialize in the biocatalytic synthesis of DNA and RNA. It can replicate the DNA from the 5′ to 3′ end with the assistance of a DNA template, primers, and deoxy-ribonucleoside triphosphates (dNTPs). PCR is the most conventional DNA technology and regarded as the "golden standard" method for DNA amplification due to its ultrasensitivity and rapidity [32]. For example, asymmetric PCR was performed to produce massive 5′-biotin-labeled target single-stranded DNA (ssDNA) of *Staphylococcus aureus*, and the PCR products were then

Table 1 Various ECL biosensors integrated with typical DNA signal amplification technologies

Amplification methods		Targets	Strategy	LOD	Ref
Polymerase	PCR	ssDNA	Asymmetric PCR and the products are hybridized with accumulated $Ru(bpy)_3^{2+}$ probes	2 pM	[15]
	RCA	PEDV antibody	RCA and the tandem repeat products are assembled with Ru-DNA nanotags	0.05 pg mL^{-1}	[16]
	HRCA	ochratoxin A	HRCA and the resultant dsDNA products are intercalated with $Ru(phen)_3^{2+}$ molecules	0.02 pg mL^{-1}	[17]
Endonuclease	NEase	miR-21	Nb.BbvcI-cleaved Y junction structure for target recycling amplification	10 aM	[18]
	NEase	miR-21	Nt.BsmAI-assisted cycling strand displacement amplification	3.3 aM	[19]
	DSN	miR-107	DSN-released target from DNA/RNA duplexes for recycling amplification	9.4 aM	[20]
Exonuclease	Exo I	Pb^{2+}	Exo I-mediated digestion of Pb^{2+}-aptamer complex for target recycling amplification	0.04 pM	[21]
	Exo III	NF-*k*B p50	Target-modulated proximity hybridization and Exo III-powered recycling amplification	29 fM	[22]
	T7 Exo	miR-155	T7 Exo-digested DNA/RNA duplexes for target recycling amplification	0.83 fM	[23]
	$RecJ_f$ Exo	TB	$RecJ_f$ Exo-mediated digestion of TB-aptamer complex for target recycling amplification	0.23 pM	[24]
	λ-Exo	miR-21	K-junction structure and λ-Exo-mediated exponential signal amplification	0.033 fM	[25]
DNAzyme	Mn^{2+} specific DNAzyme	GSH	Mn^{2+} as a substitute target triggered DNAzyme-assisted cleavage-cycling amplification	0.44 μM	[26]
Nonenzymic TSDA	HCR	ssDNA	In situ HCR and the obtained dsDNA grooves are intercalated with $Ru(phen)_3^{2+}$ molecules	15 fM	[27]
	CHA	MUC1	Target-induced CHA to circularly graft Ru-HPNSs tags on the electrode surface	0.31 fg mL^{-1}	[28]
	Entropy beacon	ssDNA	Programmable DNA cyclic amplification driven by entropy force	40 aM	[29]
DNA aggregates	Dendrimers	LPS	Self-assembled tetrahedron DNA dendrimers intercalated with Dox-ABEI probes	0.18 fg mL^{-1}	[30]
	Hydrogel	miR let-7a	Target-induced nonlinear HCR to form DNA hydrogel for PTC-DEDA intercalation	1.49 fM	[31]

immobilized with $Ru(bpy)_3^{2+}$ probes via DNA hybridization to generate an amplified ECL signal for sensitive genosensing [15, 33]. However, the PCR process requires high-precision thermal cycling; thus, sophisticated equipment, laboratory setting, and trained personnel are necessary [34]. Moreover, the PCR results might be false-positive due to the existence of nonspecific amplification [35]. Therefore, these drawbacks restrict the practical use of PCR in the ECL realm and promote the prosperous development of facile DNA amplification techniques with isothermal operation.

As a classic isothermal amplification technique without precise thermal cycling involving, RCA is a preferable method for low-cost and simple-operation assays [36, 37]. RCA process requires a circular template and a single primer to linearly amplify DNA in the presence of polymerase, producing tandem repeats complementary to the circular probe [38]. The tandem repeats with special units can be grafted with or in situ form indicators, thereby outputting the altered ECL signals. Forming G-quadruplex/hemin probe is a general method based on the conjunction of hemin and the G-quadruplex units of RCA products [39]. Zhang et al. fabricated an "off–on" switching ECL genosensor via cascading DNAzyme-induced target recycling and RCA. The massive guanine-rich (G-rich) RCA products were coupled with hemin to turn on the ECL signal of luminol/H_2O_2 system [40]. Zhuo's group realized trace Mucin 1 (MUC1) and Hg^{2+} detection via the combination of cleaving enzyme-mediated target recycling and RCA, and the in-situ formation of abundant G-quadruplex/hemin complexes triggered the amplified ECL signals [41, 42]. Besides, Ru (II) complexes, as commercial ECL luminophores, are usually used to label DNA to form functional signal probes (Ru-DNA nanotags), which can specifically hybridize with the tandem repeats of RCA products. An ECL platform was established to detect porcine epidemic diarrhea virus antibody with RCA for signal amplification, and Ru-DNA nanotags were hybridized for strong ECL emission [16]. Also, ECL-active quantum dots (QDs) are exploited, such as carbon dots (CDs), polymer dots (PDs), etc. A meticulous ECL device for human IgG assay was manufactured based on origami paper integrated with RCA, generating a linear concatenated DNA molecule to tag massive CDs for ECL readout [43]. Wang et al. established a true-color ECL imaging platform for multicomponent immunoassay with an enhanced ECL signal by coupling PDs with the RCA products [44].

In addition, the in-situ generation of efficient luminophores or quenchers can avoid tedious bio-functionalization and separation process. Chen et al. detected trace microRNA (miR)-21 through synchronizing target recycling with RCA to produce cytosine-rich (C-rich) DNA sequences, which captured Ag^+ to form Ag nanoclusters as ECL emitters by in-situ EC reduction [46]. Wang et al. utilized the target-initiated RCA products with T-T pairs to specifically interact with Hg^{2+}, which had opposite effects on the ECL of *N*-(aminobutyl)-*N*-(ethylisoluminol) (ABEI) and g-C_3N_4. Thus, a potential-resolved ratiometric ECL genosensor with the g-C_3N_4/ABEI/Hg^{2+} substrate was proposed for miR-133a determination (Fig. 1) [45].

HRCA, as an evolved version of RCA, not only inherits the advantage of isothermal expansion, but also shows higher sensitivity because of its exponential growth model [47, 48]. HRCA requires a second primer to trigger a successive cascade of primer extension and chain displacement, then generating accumulated ssDNA and double-stranded DNA (dsDNA) with various lengths [48]. Ru (II) complex, an efficient and stable ECL beacon, can be directly embedded in the grooves of dsDNA products, affording a convenient amplification process. Lin's group fabricated two ECL biosensors integrated with HRCA, and the resultant dsDNA products were intercalated with $Ru(phen)_3^{2+}$ for

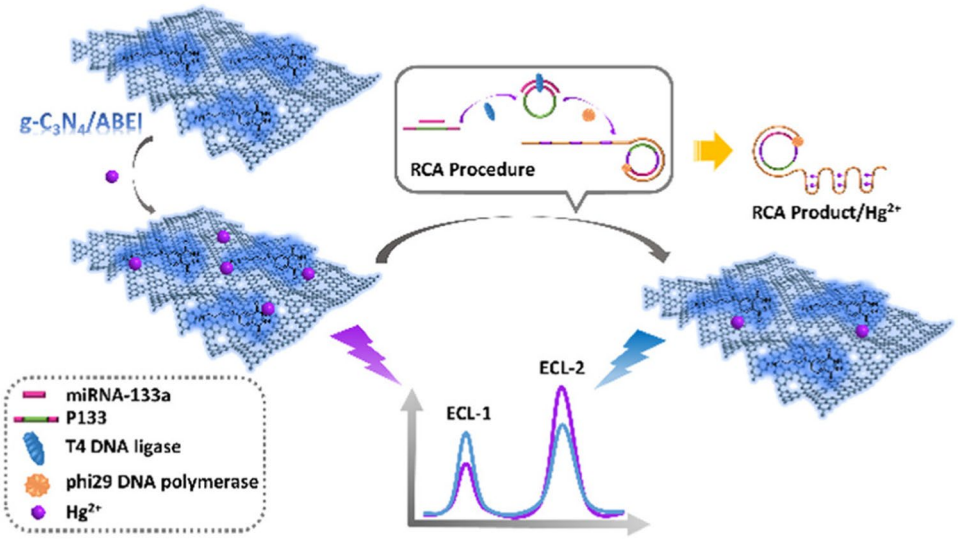

Fig. 1 Schematic illustration of g-C_3N_4/ABEI/Hg^{2+}-based and RCA-assisted strategies in proposed ratiometric ECL bioassay for miR-133a determination. Reproduced with permission from Ref. [45] Copyright 2020, American Chemical Society

ultrasensitive detection of ochratoxin A and thrombin (TB), respectively [17, 49]. This group also proposed a dual signal amplification strategy by cascading cleaving enzyme-assisted target recycling and HRCA, and the intercalated $Ru(phen)_3^{2+}$ served as ECL readout to indicate the amount of p53 DNA sequence [50]. The sensitivity of most RCA or HRCA-based ECL biosensors is significantly improved, showing great potential in clinical diagnosis, environmental monitoring, and biomedical research.

3 Cleaving Enzyme-assisted Amplification in ECL Bioanalysis

Cleaving enzymes can recognize a specific base or sequence of nucleotides and then cleave the phosphodiester bonds of nucleic acids. Cleaving enzyme-assisted target recycling amplification usually utilizes their unique cleavage capacities to release the target. The released target is recycled to be captured by an unreacted substrate for the next round. In principle, a single target can undergo N times of capture and release, thereby allowing N times of signal amplification. In this part, we outline ECL biosensing systems regarding signal amplification strategies with the assistance of two main cleaving enzymes of endonuclease and exonuclease.

3.1 Endonuclease-assisted Amplification in ECL Bioanalysis

Nicking endonuclease (NEase) is the most commonly used endonuclease in the ECL realm, which preferentially hydrolyzes only one strand of dsDNA at a specific recognition site [51]. For example, through partial integration of NEase (Nb.BbvcI)-assisted amplification strategy, several elaborated biosensors were successfully fabricated for the ultrasensitive detection of miR-21, insulin, and tumor exosomes [18, 52, 53]. Some groups further synchronized NEase (Nb.BbvcI or Nt.BstNBI)-assisted target recycling with RCA or HRCA to sensitively detect protein or specific ssDNA [41, 50, 54]. Coupled with efficient metal–organic frameworks (MOF)-based ECL emitters of Ru-polyethyleneimine (PEI)@ZIF-8 and AuNPs-PEI@Ru-PCN-777, cyclic amplification process mediated by NEase (Nb.BbvcI) was also adopted for telomerase activity and MUC1 evaluation, respectively [55, 56].

Artificial molecular machines can move and function at a single molecular level, attracting considerable attention recently [58, 59]. DNA, featuring specific base pairing, programmable operation, and divinable assembly, is particularly suitable to construct DNA machines [60]. Among these, nucleic acid-functionalized nanoparticles (NPs) can assemble 3D walking machines which are basically powered by NEase with higher payload release efficiency and superior signal amplification. For example, Tu et al. designed a 3D nanomachine powered by target miR, two hairpin DNAs, and NEase (Nb.BbvcI) to generate enormous AgNPs-tagged ssDNAs. The mimic targets not only released the ferrocene (Fc)-DNA quenching probe, but also introduced AgNPs as a coreaction accelerator, achieving a strong ECL signal of 1-pyrenecarboxaldehyde dots@mesoporous silica xerogel for miR-126 detection [61]. Wang et al. designed a bipedal 3D DNA walking machine powered by NEase (Nt.BsmAI) to convert trace target (miR-141) into substitute dopamine (DA) probes, which significantly quenched the ECL of Ce^{3+}-$Ru(dcbpy)_3^{2+}$ nanostructured coordination polymer/$S_2O_8^{2-}$ system (Fig. 2) [57]. In addition, electrode interface-derived DNA walking machine can simplify the separation and capture process. Chen et al. designed a NEase (Nt.AlwI)-powered DNA walking machine for sensitive genosensing. In their work, target ssDNA served as a walker, and the REase assisted the walker to move automatically and release the $Ru(bpy)_2$phen-containing stators from the electrode, causing a significant decrease in the ECL signal [62]. Pan et al. confined Cu nanoclusters in a porous poly-$_L$-cysteine film via in-situ EC reduction with efficient solid-state ECL emission. The cascade of alkaline phosphatase (ALP)-initiated click chemistry and NEase (Nb.BbvcI)-powered DNA walker resulted in the release of Fc-DNA from the electrode surface, and the ECL of Cu nanoclusters was recovered for ALP detection [63].

Strand displacement amplification (SDA) is an important DNA technology to improve the sensitivity of ECL bioanalysis due to its high efficiency, adaptability, and simple operation [64]. Here, we mainly introduce the SDA benefiting from the NEase's unique characteristic, which can recognize and hydrolyze the nicking site of only one strand of dsDNA. Then DNA polymerase helps to extend the ssDNA from 3′-end of the nicking site and form a new complementary strand, thereby displacing the original ssDNA. In this way, the nicking site is renewed, cleaved by NEase, and displaced cyclically to output accumulated ssDNA [65]. Chen et al. utilized this SDA strategy with the assistance of NEase (Nt.BsmAI) and DNA polymerase (phi29) for miR-21 detection. Amounts of auxiliary ssDNA were created to hybridize with the capture and Fc-tagged ssDNAs, forming a self-quenching ternary "Y" structure with a reduced ECL signal [19]. Through a similar SDA strategy, massive reporter ssDNAs were produced to straighten the hairpin DNA structure, resulting in the labeled quenching probes (AuNPs) away from the AgNPs-3D networks/CdSe QDs substrate, and the ECL signal was recovered accordingly for genosensing [66]. Lei et al. synchronized a circular peptide-DNA nanomachine with NEase (Nt.BbvCI)-assisted SDA to output mimic target, which released Fc-DNA from the electrode surface. Thus, ECL signal of the SnS_2 QDs/$S_2O_8^{2-}$/Ag nanoflower (NFs) system was turned on for cytomegalovirus pp65 antibody [67]. Moreover, the two-stage or multi-stage SDA

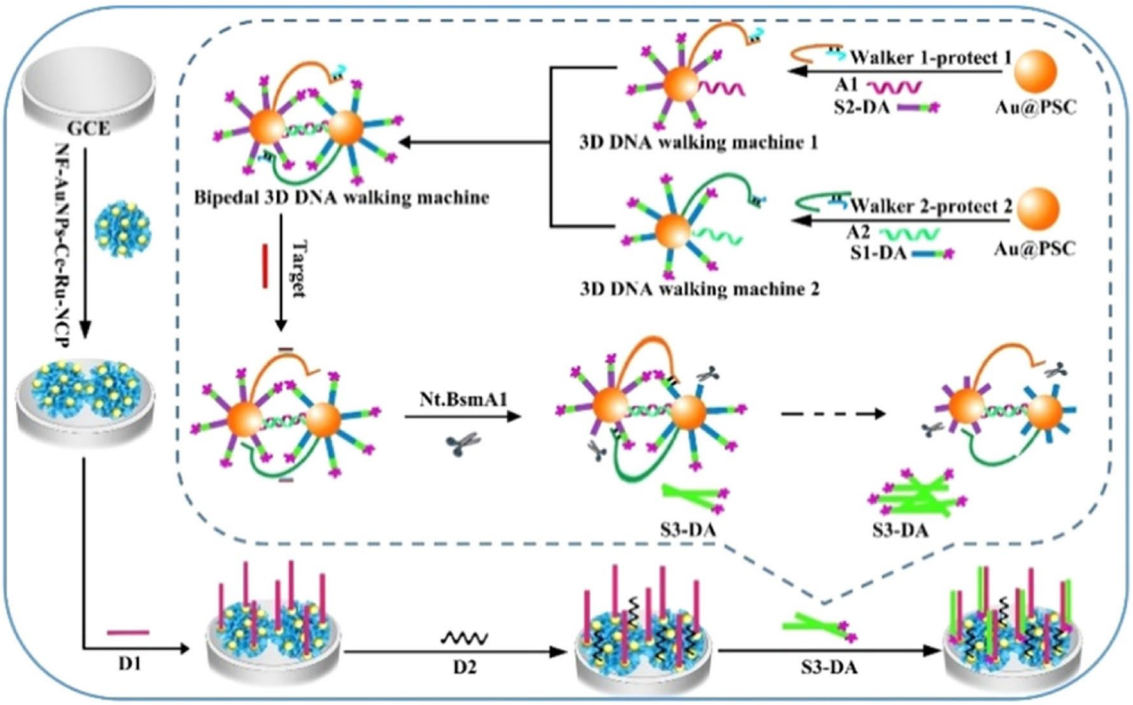

Fig. 2 Construction of the ECL biosensor and assembly process of the bipedal 3D DNA walking machine. Reproduced with permission from Ref. [57] Copyring 2020, American Chemical Society

processes were well cascaded and coordinated to achieve ultrasensitive ECL gene detection [68, 69].

Duplex-specific nuclease (DSN) is a thermostable and nonspecific endonuclease, which shows a strong preference for cleavage of dsDNA or DNA strand in DNA-RNA hybrids, but has little activity on ssDNA, ssRNA, or dsRNA [70]. Benefiting from this unique property, DSN is mostly employed for miR signal-amplifying detection via the formation of DNA-RNA heteroduplexes with a capture DNA probe [71]. For example, DSN was utilized to recycle the trace target miR and establish a distance-dependent resonance energy transfer (RET) system. The presence of target miR led to ECL quenching of CdS NCs and ECL increasing of luminol, respectively. Thus, a ratiometric ECL platform was established for accurate and sensitive quantification of miR-21 [72]. DNA bio-gate blocked the $Ru(bpy)_3^{2+}$ in mesoporous silica NPs, and the release of $Ru(bpy)_3^{2+}$ was triggered by the target miR for ECL reading, accompanying with a DSN-assisted target recycling process [73]. Combined with the DSN-mediated target recycling strategy for signal amplification, two wavelength-resolved radiometric biosensors were fabricated for sensitive and accurate determination of miRs, based on two respective ECL-RET pairs of Au-g-C_3N_4 nanosheet (NSs)/$Ru(bpy)_3^{2+}$ (Fig. 3) and AuNPs-luminol-layered-double-hydroxides/Au nanoclusters [20, 74]. A nanopore-based ECL sensor was also integrated with the DSN-assisted target recycling strategy for miR-107 assay. During the process, the specific target RNA-DNA binding and DSN cleaving detached the AuNPs-labeled DNA capture, exposing the nanopore electrode for enhanced ECL signal [75].

3.2 Exonuclease-assisted Amplification in ECL Bioanalysis

Exonuclease is a series of enzymes that can specifically hydrolyze 3,5-phosphodiester bonds and degrade nucleotides stepwise from the end of the polynucleotide chain. Utilizing specific catalytic activity and complete dissociation ability, many exonucleases have been applied to develop sensitive ECL biosensors.

Benefiting from the feature of preferentially cleaving ssDNA into nucleotides in the 3′ to 5′ direction, Exonuclease I (Exo I) is mainly integrated into aptasensor to directly release the captured target for recycling [76]. For instance, Exo I released chloramphenicol (CAP) from its aptamer for target recycling. The depletion of aptamers blocked the ECL enhancement of CdS nanocrystals (NCs) by the AuNPs-horseradish peroxidase (HRP)-linked polymer, causing a decreased ECL signal for CAP monitoring [77]. Inspired by the discovery that Hg^{2+} could efficiently inhibit the ECL of ABEI, a switchable ECL aptasensor integrated with the Exo I-assisted target recycling process was proposed for simultaneous Hg^{2+} and MUC1 assay [78]. Our group achieved trace

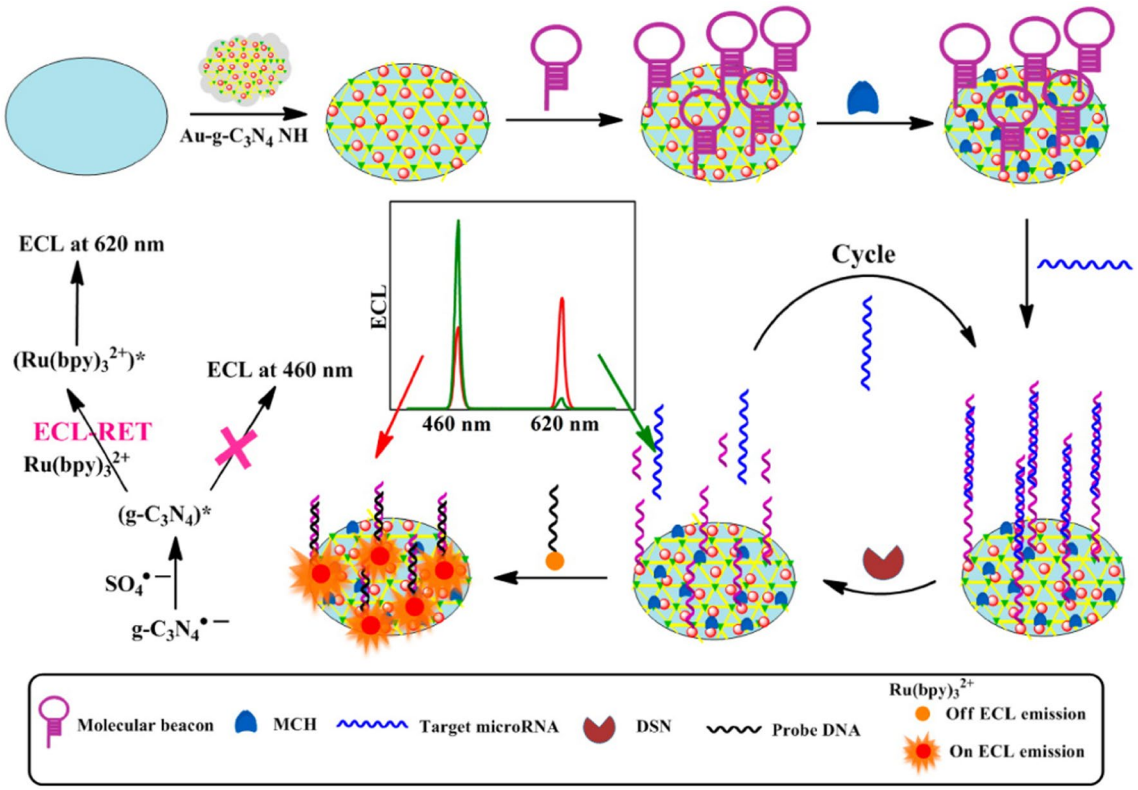

Fig. 3 Schematic illustration of the dual-wavelength ratiometric ECL-RET biosensor. Reproduced with permission from Ref. [74] Copyright 2016, American Chemical Society

Pb^{2+} quantification based on efficient ECL-RET system between g-C_3N_4 nanofibers and $Ru(phen)_3^{2+}$ implemented with Exo I-assisted decomposition of Pb^{2+}-aptamer complex for target recycling [21].

Exonuclease III (Exo III) can degrade many types of phosphodiester bonds in dsDNA, one of its main catalytic activities focuses on the release of nucleotides from 3′-OH terminal of dsDNA. DNA nanomachines powered by Exo III were designed for the transferring of trace targets into enormous reporter ssDNAs. They could further graft the doxorubicin (Dox)-ABEI probes or release the Fc probes, exhibiting recovered ECL signals for laminin and amyloid-β detection, respectively [79, 80]. Through the target-induced proximity hybridization and Exo III-assisted recycling strategy, $Ru(bpy)_3^{2+}$-nucleotides were released into a homogeneous solution and easily diffused to the indium tin oxide (ITO) electrode surface, resulting in an enhanced ECL signal for transcription factor (NF) assay (Fig. 4) [22]. Meanwhile, the CdSe QDs bilayers/Au@Ag-based ECL-RET system was established for sensitive detection of TB via an autocatalytic multiple amplification strategy, containing two Exo III and one endonuclease-aided recycling processes [81].

T7 exonuclease (T7 Exo) has been reported to hydrolyze dsDNA or DNA/RNA helixes in the 5′ to 3′ direction without requiring a specific recognition site in the target sequence, but it cannot decompose either dsRNA or ssRNA [23]. Zhang et al. utilized T7 Exo to digest the RNA/DNA hybrids and recycle the target to form 3D DNA skeletons. AgNPs were then formed by in-situ reduction and electrostatic adsorption, which extraordinarily enhanced the ECL of graphene QDs/aminated 3,4,9,10-perylenetetracarboxylic acid (PTCA)/Au@Fe_3O_4 substrate for miR-155 quantification [23]. Li et al. proposed an efficient target conversion strategy for MUC1 assay via target and mimic target synchronous recycling amplification with the assistance of T7 Exo. Then, massive mimic ssDNAs were generated to trigger the in-situ formation of DNA NFs, which were further loaded with abundant Dox-ABEI for extremely high ECL readout [82]. Yang et al. introduced an antibody-powered triplex-DNA nanomachine to release the cargo ssDNA, which further participated in a T7 Exo-assisted strand displacement cyclic process to release the Fc quenching probes. Hence, the ECL of rubrene microblocks/dissolved O_2/porous palladium nanospheres ternary system was recovered for anti-digoxigenin detection [83]. Nie et al. manufactured a T7 Exo-assisted DNA walking machine, which could cyclically release Zn-doped MoS_2 QDs from the reductive Cu (I) particles substate, exhibiting a decreased ECL signal for human papilloma virus 16 DNA detection [84].

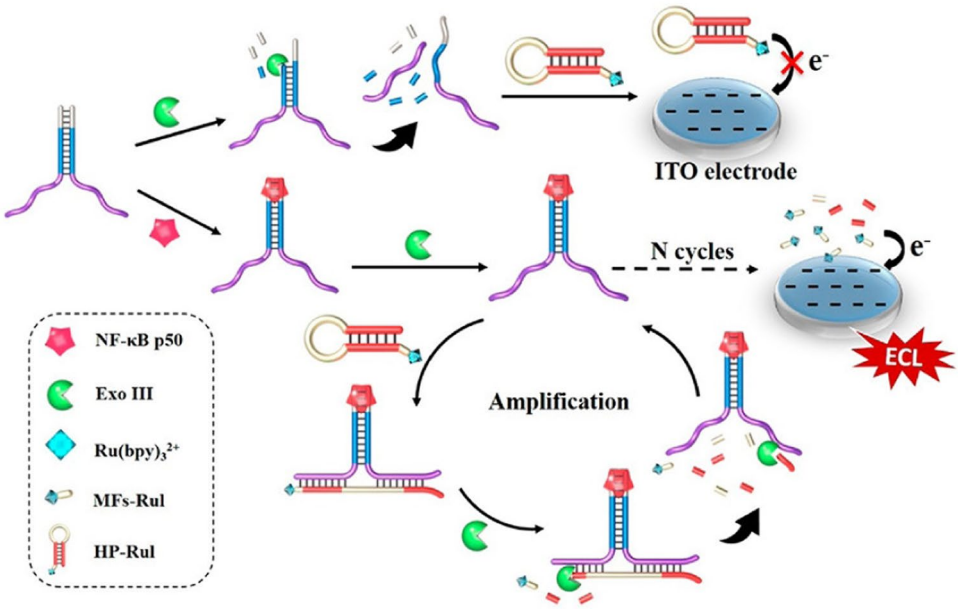

Fig. 4 Homogeneous ECL biosensor for NF assay using target-modulated proximity hybridization and Exo III-powered signal amplification strategy. Reproduced with permission from Ref. [22] Copyright 2020, American Chemical Society

Additionally, $RecJ_f$ exonuclease ($RecJ_f$ Exo) can specifically catalyze the decomposition of ssDNA into deoxynucleotide monophosphates in the direction from 5′ to 3′-terminal, and it was reported to release target TB and alpha-fetoprotein (AFP) from their aptamers for target recycling, improving their detection sensitivity accordingly [24, 85]. Lambda exonuclease (λ-Exo) can progressively hydrolyze dsDNA into nucleotides from 5′-PO_4 terminal. With the assistance of λ-Exo and the designed "K" DNA structure, Li et al. proposed an exponential amplification strategy for miR-21 quantification, and ten cells could be detected in human lung cancer cell lines [25].

4 DNAzyme-assisted Amplifications in ECL Bioanalysis

DNAzyme is a functional DNA molecule, exhibiting catalytic activity toward a specific substrate strand, which usually contains a single RNA linkage (rA) as the embedded cleavage site [86]. After incubating with target metal ions (e.g., Zn^{2+}, Pb^{2+}, Cu^{2+}, Mn^{2+}, etc.), DNAzyme forms and cleaves the rA, causing the splitting of substrate strand [87]. Since metal ions are vital and specific to trigger the catalytic activity, various DNAzymes directly serve as sensing platform for metal ions, bypassing the requirements of metal immobilization [88]. Liang et al. constructed DNAzyme micronet to load large amounts of $Ru(dcbpy)_2dppz^{2+}$ on the electrode surface, which could be circularly decomposed by the target Pb^{2+} to release the luminophores, causing a decreased ECL signal for ultratrace Pb^{2+} analysis [89]. Because GSH could reduce MnO_2 NSs to Mn^{2+}, Ge et al. measured GSH using substitute Mn^{2+}-specific DNAzyme and NEase (Nt.BbvCI)-powered DNA walker to form allosteric streptavidin (SA) aptamers for the capture of CdS:Mn QDs-SA ECL tags [26].

DNAzyme probes are also designed for genosensing due to their remarkable catalytic activity and stability. Zhou et al. designed an Mn^{2+}-specific DNAzyme-induced autonomous walking machine, prompting the formation of A-T riched dsDNA on the top of tetrahedral DNA nanostructure for Cu^{2+} capture. Cu nanoclusters were formed subsequently via in-situ EC reduction, exhibiting remarkable ECL for ultrasensitive miR-155 quantification [90]. Ling et al. combined the self-enhanced CuMn-CeO_2-PEI-luminol ECL emitter with the Mg^{2+}-specific multicomponent DNAzyme target recycling strategy to sensitively analyze DNA, which was extracted from *Group B Streptococci* strain [91]. Wang et al. cascaded Pb^{2+}-specific DNAzyme-assisted target recycling process with a 3D DNA nanomachine, which inhibited the multiple ECL-RET between Alexa fluor and PtNCs@$Ru(dcbpy)_3^{2+}$, leading to significantly decreased ECL intensity for miR-141 assay [92].

Aptazyme, a combination of DNAzyme and aptamer, provides a new approach for sensitive protein detection. For example, Zn^{2+}-specific aptazyme for both target recognition and recycling was employed as one of the signal amplification modules for TB detection [93, 94]. Besides, the DNAzyme can also be utilized as the signal amplification tags to indicate immunoassay. Through sandwich-type immunoreaction of target cardiac troponin I (cTnI) and Pb^{2+}-specific DNAzyme, accumulative DNA walkers were generated to start the walking machine and trigger the ECL recovery [95].

5 Toehold-mediated DNA Strand Displacement-assisted Amplification in ECL Bioanalysis

These DNA amplification technologies discussed above require the participation of enzymes, which show inherent shortcomings of high experimental cost and susceptibility to environmental conditions, such as temperature, acidity, and alkalinity. Hence, novel nonenzymic DNA amplification technologies have attracted widespread attention and are in high demand. TSDA, pioneered by Yurke et al. [96], is considered to be an efficient signal amplification approach without the enzyme involvement, and it can even achieve polynomial or exponential growth [97]. Toehold refers to an ssDNA fragment consisting of 5–8 bases, suspended at the sticky end of dsDNA, where strand displacement can be initiated by branched strand migration. TSDA is triggered by the hybridization of fuel chain with the complementary toeholds of two or more pre-hybridized strands and processed via a chain exchange mechanism to displace the original short ssDNA, which was driven thermodynamically by entropy in a spontaneous process [98]. Hybridization chain reaction (HCR), catalytic hairpin assembly (CHA), and entropy beacon as classical TSDA methods are increasingly applied to ECL bioanalysis.

5.1 Hybridization Chain Reaction

Since the first report published by Dirks and Pierce in 2004 [99], HCR has become an important signal amplification strategy. Typically, the target ssDNA initiates continuous hybridization with two hairpin DNA containing the toehold areas and results in the polymerization of oligonucleotides to form long-nicked dsDNA polymers under mild conditions [100]. Because this process can achieve simultaneous target identification and signal amplification, HCR has been widely integrated into ECL biosensing systems [101, 102].

Ru (II) complexes can be directly embedded into the grooves of the long-necked dsDNA for ECL readout. Lu et al. constructed a Faraday cage with a large specific surface area and excellent electron transport property via anchoring HCR products on graphene oxide (GO) surface and then embedding $Ru(phen)_3^{2+}$ as the ECL transducer to detect miR-141 [103]. Huang et al. combined specific Cu^+-catalyzed azide-alkyne cycloaddition (click chemistry) and the highly efficient HCR with $Ru(phen)_3^{2+}$ beacon in a homogeneous solution for pyrophosphatase evaluation [104]. Zhang et al. prepared $Ru(bpy)_2(cpaphen)^{2+}$-intercalated HCR products on the electrode surface with a strong initial ECL signal, and the GSH-reduced Mn^{2+} served as the coenzyme factor to release the luminescent composites, resulting in a weak ECL signal for GSH detection (Fig. 5) [105]. Li et al. synthesized silver-based metal–organic gels as a coreactant substrate for the $Ru(phen)_3^{2+}$-intercalated HCR products, displaying a significantly enhanced ECL signal for genosensing [106].

Certain metal ions with catalytic capability can be inserted into special base pairs of HCR products. Lei et al. embedded Ag^+ into C-rich HCR products as a powerful coreaction accelerator to enhance the PTCA/$S_2O_8^{2-}$ ECL system, eventually realizing the detection of Hg^{2+} [107]. Based on the enhancing and quenching effects of the HCR products embedded with Ag^+ and Ag nanoclusters (in-situ

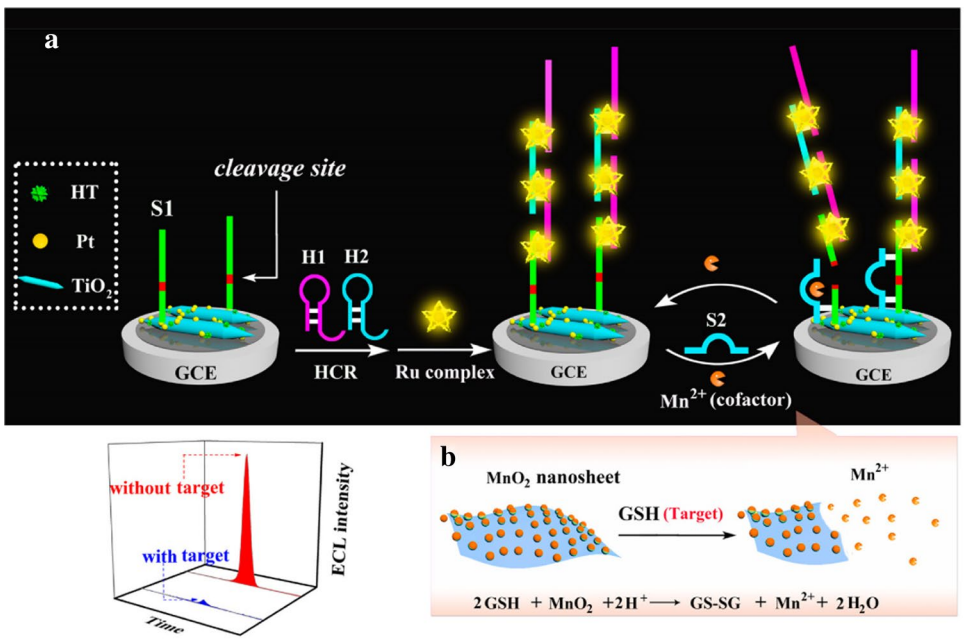

Fig. 5 **a** The preparation process of the proposed ECL biosensor; **b** the conversion process of GSH into substitute target Mn^{2+}. Reproduced with permission from Ref. [105] Copyright 2019, American Chemical Society

reduction) toward the CdSe QDs/$S_2O_8^{2-}$ ECL system, respectively, a switchable ECL platform was established for sequential TB and miR-21 measurement [108]. Cu^{2+} was intercalated into A-T complementary bases of HCR products and converted to Cu nanoclusters by in-situ EC reduction, which served as efficient ECL emitters for sensitive miR-21 detection [109].

Catalytic hemin/G-quadruplex DNAzyme can be formed directly with hemin and the G-rich HCR products. Highly sensitive evaluation of adenine methyltransferase (Dam MTase) activity was accomplished based on the fact that REase (Nt.AlwI) was unable to cleave the Dam MTase-methylated dsDNA, and it further inhibited the formation of the HCR-based DNAzyme, resulting in a drastically enhanced ECL signal of O_2/$S_2O_8^{2-}$ system [110].

Nanocarriers can also be tagged on HCR products for high luminophores payload. For example, by utilizing AuNPs-luminol labeled hairpin DNAs for HCR, the long dsDNA polymers were fully loaded with signal molecules, outputting strong ECL emission. This strategy has been successfully proposed for cytosensing based on a "one-pot" cascaded DNA automachine and a paper-based closed-bipolar electrode, respectively [111, 112]. Novel emitters of europium multiwalled carbon nanotubes with carboxyl groups were also introduced to NH_2-terminated HCR products via amidation reaction and emitted an amplified ECL signal for trace TB detection [24].

Loading quenching probes is an alternative way to access the bioanalysis. Our group synthesized a dual-potential ECL emitter of $CsPbBr_3$@hollow g-C_3N_4 nanosphere (HCNS). The anodic ECL of $CsPbBr_3$ was quenched by the HCR products-rhodamine 6G due to the efficient ECL-RET, while the cathodic ECL of HCNS remained unchanged. Further combining with a well-designed DNA probe, a ratiometric strategy was proposed for the sensitive and accurate evaluation of CD44 expression on MCF-7 cells [113]. Wang et al. measured C-peptide by utilizing a NEase (Nb.BvCI)-powered walking machine to produce intermediate ssDNA, which further initiated the HCR for DA loading, and the massive DA exhibited a dual quenching effect to the ECL of Ru-PEI-ABEI [114]. Ultrasensitive miR-21 detection was also realized by anchoring enormous Fc on the sensing surface via the target-initialed HCR self-assembly, and the Fc quenched the ECL of luminol/dissolved O_2 system with coreaction accelerators of ZnO nanostars and MnO_x microflowers, respectively [115, 116].

5.2 Catalyzed Hairpin Assembly

As a DNA circuit without enzyme participation, CHA can handily proceed with a hairpin DNA probe through self-assembly and disassembly of original ssDNA [117]. Therefore, CHA has been frequently designed for signal-amplifying ECL bioanalysis. Sensitive and accurate gene quantification helps to understand human genetic diseases and other related biological activities. Sun et al. synthesized flowerlike MoS_2/GO/o-MWNTs nanohybrids as the electrode substrate for the immobilization of Cu-Zn-In-S NCs emitters. Using an Au nanostars-labeled hairpin DNA probe, CHA-induced target recycling promoted the establishment of a near-infrared ECL-RET system for FLT3 gene detection [118]. Feng et al. grafted glucose oxidase (GOD) on the vertex of the DNA scaffold through the CHA-assisted target recycling process. Then, the GOD catalyzed glucose to form H_2O_2, which efficiently quenched the ECL of Ru(bpy)$_3^{2+}$-tripropylamine (TPrA) system for genosensing [119].

MiRs, a family of endogenous noncoding RNAs about 18–25 bases in length, have been recognized as promising biomarkers. Yu et al. amplified ECL readout for miR-21 assay by coupling CHA-mediated target recycling strategy with ECL system of Ru(dcbpy)$_3^{2+}$ with PEI and thiosemicarbazide as both intramolecular and intermolecular coreactants [120]. Zhang et al. recycled the target miR-21 using three pairwise and partly complementary hairpin DNA probes to form a "Y" structure via CHA, and massive reporter ssDNAs were then produced via Pb^{2+}-specific DNAzyme. After that, a designed DNA tweezer was triggered by the reporter ssDNAs, situating an ECL-RET state to indicate the ECL signal. Besides, the state could be regenerated more than seven times by a simple strand displacement [121]. Jiang et al. designed an elaborate DNA nanomachine involving CHA-mediated target recycling process, producing massive Fc-DNA probes to quench the ECL of g-C_3N_4 NSs for miR-21 assay [122]. Through CHA-mediated target recycling and acid dissolution processes, trace target miR-21 was converted to large amounts of substitute Zn^{2+}. Afterward, these Zn^{2+} drove a speedy DNA rolling machine to cut off the Fc-tagged track DNA, thus recovering the ECL signal of CdS:Mn QDs substrate (Fig. 6) [123]. Wang et al. fabricated a paper-based Au-bipolar electrode for miR-155 detection. The cathode is a sensing cell integrated with a typical CHA-assisted target recycling process to load AuPd NPs on the electrode surface, and the anode is a reporting cell coupled with the classical Ru(bpy)$_3^{2+}$/TPrA ECL system [124]. Luo et al. synthesized conjugated PDs as the coreactant-free emitters and proposed a dual signal amplification strategy with the CHA-mediated target recycling and Exo III-powered DNA walker for sensitive miR-155 sensing [125]. Zhu et al. cascaded the DSN-assisted target recycling and multiple CHA-related amplification processes to output massive long dsDNAs. During the process, the Fc-DNA probes were displaced and the resultant dsDNAs were further combined with Ru(phen)$_3^{2+}$, causing reduced EC and enhanced ECL signals. Based on a ratiometric ECL/EC

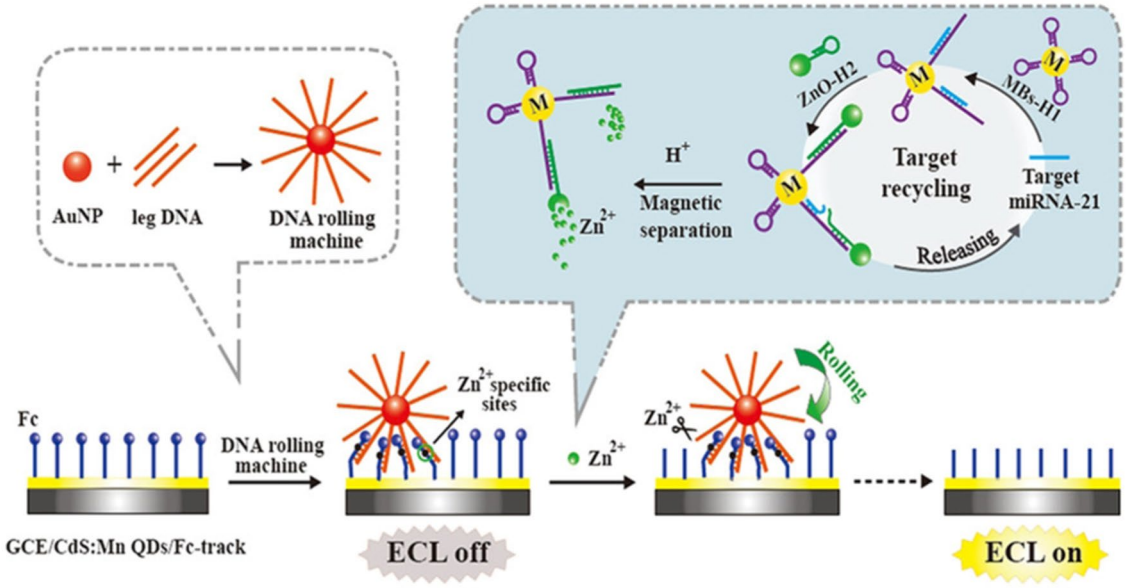

Fig. 6 Fabrication process of the ECL biosensor based on a Zn^{2+}-driven DNA rolling machine for speedy detection of miR-21. Reproduced with permission from Ref. [123] Copyright 2019, American Chemical Society

strategy, accurate and sensitive quantification of miR-499 was finally achieved [126].

Indirect protein detection based on specific aptamers can realize the conversion of target protein to nucleic acid analysis. MUC1, an important biomarker in serum or tissues, is closely related to several lethal diseases, such as pancreatic, bladder, and breast cancers [127]. Therefore, it is of great significance to design suitable DNA amplification strategies for sensitive MUC1 detection. Chen et al. synthesized hollow porous polymeric nanospheres containing self-enhanced PEI-Ru(mcbpy)$(bpy)_2^{2+}$ complex (Ru-HPNSs) with remarkable ECL efficiency. In their work, the Ru-HPNSs tags were grafted onto the electrode surface through a CHA-mediated cyclic process, achieving high ECL readout for MUC1 detection [28]. Li et al. detected MUC1 sensitively via enzyme-free target recycling with double outputs, and massive mimic ssDNAs further initiated a CHA-assisted recycling process to load the signal probes of ABEI-Ag-MoS_2 NFs [128]. Yao et al. synthesized 2D ultra-thin Zr_{12}-9,10-anthracene dibenzoate MOF nanoplate with high solid-state ECL efficiency because of the restriction of intramolecular motions. The ECL substrate was further combined with a CHA-assisted target recycling process and a bipedal walking machine to load Fc quencher on the electrode surface, resulting in a decreased ECL signal for MUC1 assay [129].

In addition, aflatoxin M1 (AFM1), a class 1 human carcinogen from aflatoxin B1, poses a serious threat to human health. Zeng et al. constructed an ECL aptasensor for trace AFM1 detection with PTCA as the ECL luminophore based on dual amplification of CHA and HCR [130]. Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative bacteria and is the main cause of many diseases, including fever, microcirculation disorder, endotoxin shock, etc. LPS could be quantified sensitively using ABEI-loaded tetrahedron DNA dendrimers as the ECL indicator and CHA-assisted cyclic strategy for signal amplification [30].

5.3 Entropy Beacon

Inspired by the entropy-driven DNA catalysis pioneered by Yurke's group [131], entropy beacon can achieve enzyme- and hairpin-free signal amplification, showing its inherent potential in high-sensitivity ECL bioanalysis [132, 133]. Feng et al. performed a programmable DNA cyclic amplification initiated with target ssDNA via entropy-driven force to produce accumulated GOD-labeled reporter ssDNAs, which were further grafted onto the top vertex of self-assembled DNA tetrahedral scaffolds. Thus, the ECL signal of $Ru(bpy)_3^{2+}$-TPrA system was quenched by H_2O_2, the product of GOD catalyzing glucose (Fig. 7) [29]. Chen et al. utilized the entropy beacon to convert target miR-21 to abundant mimic ssDNAs, which further initiated the dynamic DNA molecular machine. The free walker moved on a lipid bilayer interface to make Fc away from the $Ru(bcbpy)_3^{2+}$, affording a recovered ECL signal [134]. A similar conversion and amplification strategy via the entropy beacon was also coupled for the capture of $Ru(phen)_3^{2+}$-DNA nanoclews to the sensing electrode for miR-21 detection [135].

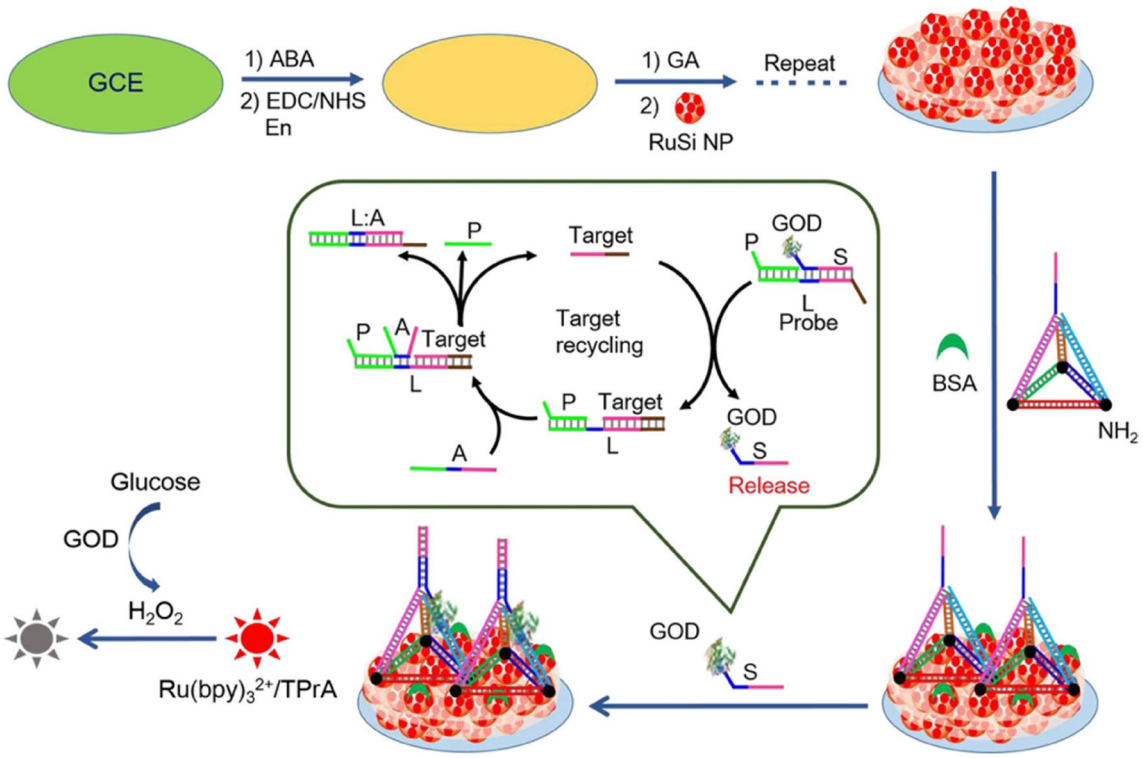

Fig. 7 Working principle of the programmable DNA cyclic amplifying ECL genosensor. Reproduced with permission from Ref. [29] Copyright 2017, American Chemical Society

6 Self-assembled DNA Aggregates-assisted Amplification in ECL Bioanalysis

As a building material with biocompatibility and eco-friendliness, DNA has been often assembled into various DNA aggregates, serving as desirable carriers to improve luminous efficiency. For instance, different types of DNA dendrimers were self-assembled as nanocarriers for the Dox-ABEI loading, affording high ECL efficiency for ultrasensitive LPS and laminin detection, respectively [30, 79]. Meanwhile, $[Ru(dcbpy)_2dppz]^{2+}$ and its coreactant of *N,N*-diisopropylethylenediamine (DPEA) were co-embedded into the DNA dendrimer to assemble the self-enhanced DNA composite, which was utilized as ECL labels for sandwiched immunoassay of *N*-acetyl-β-$_D$-glucosaminidase, a reliable biomarker for diabetic nephropathy [136]. Initiated with a trigger DNA in the hairpin switch, DNA dendrimers were self-assembled by in-situ nonlinear HCR for $Ru(phen)_3^{2+}$ intercalation, realizing label-free ECL detection of BCR/ABL fusion gene [137].

Additionally, multifarious assembles, such as DNA nanotube, DNA nanonet, 3D DNA matrix, 3D DNA network, and DNA hydrogel, are also constructed as ideal nanocarriers. Wu et al. designed a highly efficient ECL system for tryptase sensing, containing DNA nanotubes loaded with Dox-luminol as efficient ECL probes, dissolved O_2 as endogenous coreactant, and Au-Ag-Pt hetero-nanostructures as coreaction accelerator [139]. One-step self-assembled DNA nanonet was triggered by target miR-21 for Dox-ABEI embedding. Further using dissolved O_2 as coreactant and Ag_3BiO_3 NCs substrate as coreaction accelerator, high-intense ECL of ABEI for miR-21 assay was achieved [140]. A 3D DNA matrix was self-assembled from alkyne-rich tetrahedral DNA blocks on the electrode surface. Target GSH reduced Cu^{2+} to Cu^+, which served as the catalyst to link N_3-AuAg nanoclusters with the 3D DNA matrix. By this means, massive AuAg nanoclusters emitted strong ECL for GSH assay (Fig. 8) [138]. Zhang et al. assembled a target-triggered 3D DNA network via cyclic CHA with a high loading ability, and it was completely collapsed by dissolving AuNPs to covert trace target miR-21 to massive mimic targets for signal amplification [141]. DNA hydrogel with abundant dendritic DNA structures was generated via a target (miR let-7a)-induced nonlinear HCR, which was entrapped with abundant ECL intercalator of amphiphilic perylene derivative modified with *N,N*-diethylethylenediamine (PTC-DEDA) for an enhanced ECL intensity [31].

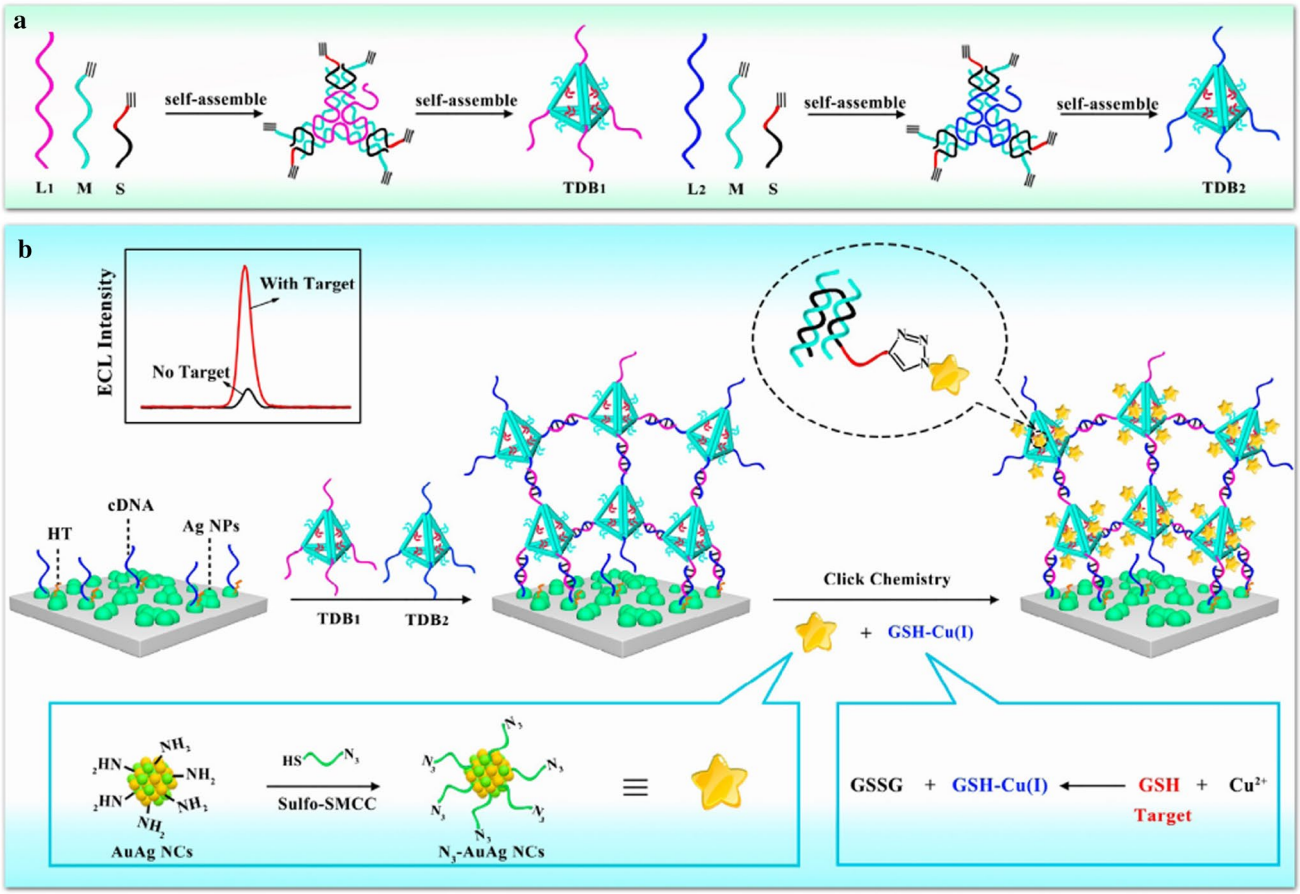

Fig. 8 Schematic illustration of the biosensor based on 3D DNA matrix for GSH detection. Reproduced with permission from Ref. [138] Copyright 2020, American Chemical Society

7 Conclusion and Outlook

The demands for ultrasensitive bioanalysis and its trend in clinical diagnosis, biomedical research, and pharmaceutical development have forced the advancement of numerous DNA amplification strategies in the ECL domain. First, PCR as the "gold standard" method for DNA amplification is rarely used in ECL bioanalysis due to the requirement of high-precision thermal cycling. Then, a wide variety of isothermal DNA amplification technologies are proposed for ECL biosensing, including RCA, HRCA, and cleaving enzyme and DNAzyme-mediated amplification. Next, considering the high experimental cost and susceptibility of enzymes, nonenzymic DNA amplification technologies are increasingly applied to ECL applications, such as HCR, CHA, and entropy beacon. Finally, self-assembled DNA aggregates feature biocompatibility and eco-friendliness, serving as nanocarriers for luminophores loading with enhanced ECL efficiency. This review provides a thorough review of the basic principles and broad applications of ECL biosensors integrated with DNA signal amplification strategies, hoping to provide a comprehensive understanding of the relevant contents and new ideas.

Despite the great potential of DNA amplification-based ECL biosensing strategies, the practical applications of such biosensors are still in their infancy. Although the cascade of multiple ones can achieve remarkable sensitivity in most cases, the accuracy is being at risk because of the increasing complicated circuits. Balancing the sensitivity and accuracy is a significant project for future progress. In our humble opinion, the lack of ready-to-use kits for performing these DNA technologies hinders their further advancement, and commercial kits are still to be developed accordingly. Nevertheless, we envision that this field will continue to grow benefiting from the coupling of integrated and miniaturized biosensing devices. Thus, it is urgent to develop convenient and budget DNA amplification circuits, which are further coupled with disposable ECL biosensors based on paper, cloth, or chip to achieve point-of-care tests and low-cost mass bioanalysis. DNA origami provides a new idea for the design of DNA assembles with special structure and function, serving as the biocompatible nanocontainer for

customized usages. In addition, ECL imaging as a burgeoning technique coupled with DNA amplification technologies might realize multi-component analysis and even achieve single-molecule sensitivity for in-situ visualized bioanalysis.

Authors' contributions YC summarized the literatures and wrote the manuscript under the instruction of CM and J-JZ.

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