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Programmed DNA walkers for biosensors

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ABSTRACT

Nowadays, due to excellent biological and polymeric characteristics, DNA has been widely noted as an emerging building block to construct diverse materials for biosensing, *in vivo* imaging, drug delivery, and disease therapy. Particularly, relying on programmability, predictability, and stability of DNA, DNA walkers have opened new and exciting opportunities in modern life sciences for target detection and biological analysis, which are constructed by self-assembly of DNA or combining DNA with other nanomaterials (*e.g.*, quantum dots, gold nanoparticles, magnetic nanoparticles, polymers). Compared with conventional nanomaterials (lanthanide-doped upconversion nanoparticles, magnetic nanomaterials, carbon dots, silicon dots, and so on), DNA walkers showed convenient modification, lower biotoxicity, excellent biocompatibility and high biostability, improving the biological application. Meanwhile, with high-speed operating efficiency and sustainable operation, DNA walkers powered by strand displacement reaction or protein enzyme/DNAzyme reaction, have highly sensitive detection and signal amplification abilities, which are applied in biosensing, material assembly and synthesis, and early cancer diagnosis. Worthily, DNA walkers could be regarded as signal amplifiers, which enhanced the signal transduction and amplified biosensor sensing signals. Herein, we systematically and comprehensively summarized the operating principles of various DNA walkers, categorized rational design of the DNA walker, and outlined the application of DNA walker in biosensors. Furthermore, the challenges and future trends of DNA walkers were discussed.

1. Introduction

DNA, classic biological molecule of life, carried genetic information and participated biological processes. Each DNA base was composed of nitrogenous bases including adenine (A), thymine (T), guanine (G) and cytosine (C); phosphate group; and 5-carbon sugars (deoxyribose). Through purine and pyrimidine base-pairing rules to construct Watson–Crick double helix, DNA could form stable double helix DNA structures [1,2]. Meanwhile, due to the biodegradability, rapid synthesis, and easy modification, DNA has developed to be an ideal building block to construct specific, stable, biocompatible, and functional DNA walkers for the application in various analytical and biological fields [3–5].

Relying on the advantage of the programmability and predictability of DNA structures, DNA walkers could be controllable nanoscale structures, resulting in that the DNA walkers exhibited excellent transportation feature for biosensing and bioimaging applications [6–9]. Nowadays, DNA walkers could be constructed by self-assembly of DNA or combining DNA with other nanomaterials (*e.g.*, quantum dots, gold nanoparticles, magnetic nanoparticles, polymers) [10–12]. In addition, through self-assembly of DNA, DNA walker was stable, modifiable, and biocompatible [13]. Understandably, the DNA walkers by combining with other nanomaterials, were endowed with the advantage of DNA and nanomaterials, presenting richer biological application [14]. Compared with traditional single-strand DNA probes showing poor cell internalization capability, the DNA walkers through self-assembly of DNA shared rigid structure to improve the biocompatibility and limit degradation [15–17]. Besides, DNA walkers as nanomaterials possessed prominent features for elevating cell internalization efficiency and enhancing resistance to enzymatic digestion [18–20].

To date, the programmable and controlled DNA walkers have emerged in various types such as 1D (1D) tracks, 2D (2D) tracks, 3D (3D) tracks, showing powerfully potential applications in biosensing, bioimaging, drug delivery, and material manufacture [21–26]. Compared with the traditional nanomaterials, the DNA

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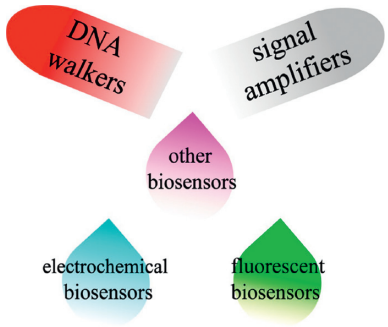

Fig. 1. Research field of DNA walkers presented in this review.

walkers were intelligent, due to precise sequences, facile modification, and controlled intermolecular interactions. After decades of development, various DNA walkers [27–34] have been explored to perform intricate tasks. Nowadays, DNA walkers were applied in water purification, sensors and medicine, exhibiting high specificity and excellent sensitivity. Noteworthily, DNA walkers could be driven by toehold-mediated strand displacement (TMSD) [35–37], or protein enzyme/DNA enzyme (DNAzyme) [38–41], leading to signal transduction and signal amplification, generating changes of the DNA structure for releasing molecules or detectable signals. These intelligent walkers enhance the sensitivity and specificity of biosensing.

As shown in Fig. 1, to be signal amplifiers, DNA walkers demonstrate great potential for developing a variety of biosensors applied in ultrahigh-density data storage and clinical diagnosis. Meanwhile, it is a challenge to design intelligent DNA walkers for performing the assigned tasks in different fields. In this review, we focused on the design of various DNA walkers applied in biosensing and bioimaging [42–46]. Firstly, we discussed the mechanisms of the DNA walker operation in detail. Secondly, we introduced the typical design of DNA walker based on the DNA self-assembly and DNA combining with nanomaterials, demonstrating the different features and advantages. Thirdly, we enumerated some respective DNA nanostructure-based probes such as 1D, 2D and 3D nanostructures, and clarified their major strategies and characteristic properties. Finally, we summarized the biomedicine and non-biomedicine applications of DNA walker. Moreover, we discussed the future prospects and challenges for the development of DNA walker in this review.

2. Principle of the walker

The brightest spotlight of the walker is to perform the desired tasks allowing precise navigation toward the targeted site, or to accelerate a recognition or catalytic process. Generally, the initial balance of the walker could be broken by external driving forces such as local fuel consumption, and light [47–49]. Meanwhile, to repair the equilibrium system, a fuel molecule is provided. Concurrently, these processes were along with the signal generation. Through the cyclic process of breaking and restoring this equilibrium, the energy cascade promoted the walker operating by the autonomous and progressive walking along tracks. Thereby, this novel operation mechanism attracted a great deal of attention in bioanalytical and non-bioanalytical chemistry. Herein, we reviewed the principle of the walker. Either the strand displacement reaction or protein enzyme/DNAzyme reaction, the DNA walkers with different driving power could be identified as signal amplifiers. Table 1 demonstrated the linear range and LOD of DNA walkers based on diverse signal amplification strategies, showing the DNA walker could realize the different target detection, such as DNA, RNA, protein, bacteria, and virus.

2.1. Strand displacement reaction

Relying on Watson-Crick hydrogen bonds, the strand displacement reaction as a key driving force, could enable the walker to govern and control strand hybridization [50]. Through toeholds-mediated strand displacement reaction, the walker could displace the prehybridized strands towards branch migration, initiating the autonomous and progressive walking. Considering that the DNA sequence length and toehold complementary regions can be altered, the strand displacement reaction-based walkers are designed in a variety of models.

It was well-known that Pierce's group first presented the concept of DNA walker in 2004 [35], through designing a synthetic transport device which could mimic the movement of motor proteins. This bipedal DNA walker provided by Pierce's group realized real-time monitoring of walker movement *via* multiplexed fluorescence quenching. As shown in Fig. 2A, the DNA walker was attached to tracks by the attachment fuel strands (A1 and A2), and moved along tracks under the external control of detachment fuel strands (D). When the D1 hybridized with A1 by the toeholds-mediated strand displacement reaction, the walking leg was released and reattached to the adjacent tracks, resulting in moving along the tracks. Through the progressive movement, this DNA walker achieved the energy conversion by strand displacement reaction with no enzymes. In addition, Xiang's group [51] described a DNA-fueled machine for monitoring low-expressed microRNA (miRNA) in live cells (Fig. 2B). In the presence of target miRNA, the walker could release hairpin sequences from the dsDNA-conjugated gold nanoparticles. Through two cascaded strand displacement reactions, the DNA fuel strands assisted the recycling of the target miRNA sequences, resulting in the signal amplification. Obviously, this persistent walking mode driven by the strand displacement reaction required the addition of specific fuel strands, which leaded to the stable "trap state" formation. To prevent "trap states" formation, a metastable hairpin structure was provided as

Table 1
Linear ranges and LODs of the biosensors.

Design principles of DNA walker	Analytes	Linear range	LOD	Ref.
Strand displacement reaction	TK1 mRNA	0.01–40 nmol/L	4.2 pmol/L	[35]
Strand displacement reaction	let-7a miRNA	5 pmol/L–1 nmol/L	1.7 pmol/L	[51]
Strand displacement reaction	let-7a miRNA	10 pmol/L–200 nmol/L	6 pmol/L	[53]
Protein enzyme reaction	APE1	1×10^{-5}–0.1 U/μL	5.54×10^{-6} U/μL	[59]
Protein enzyme reaction	miRNA-182–5p	0.1 fmol/L–1 nmol/L	31.13 amol/L	[74]
Protein enzyme reaction	miRNA 21	10 fmol/L–5 nmol/L	5.2 fmol/L	[70]
Protein enzyme reaction	S. typhimurium	$10–10^4$ CFU/mL	4 CFU/mL	[87]
DNAzyme reaction	miRNA 21	0.2–1000 pmol/L	97 fmol/L	[55]
DNAzyme reaction	miRNA-10b	5–200 pmol/L	1 pmol/L	[60]
DNAzyme reaction	survivin mRNA	0.7 pmol/L–4 nmol/L	0.51 pmol/L	[68]
DNAzyme reaction	miRNA-21	50 pmol/L–6 nmol/L	10 pmol/L	[81]

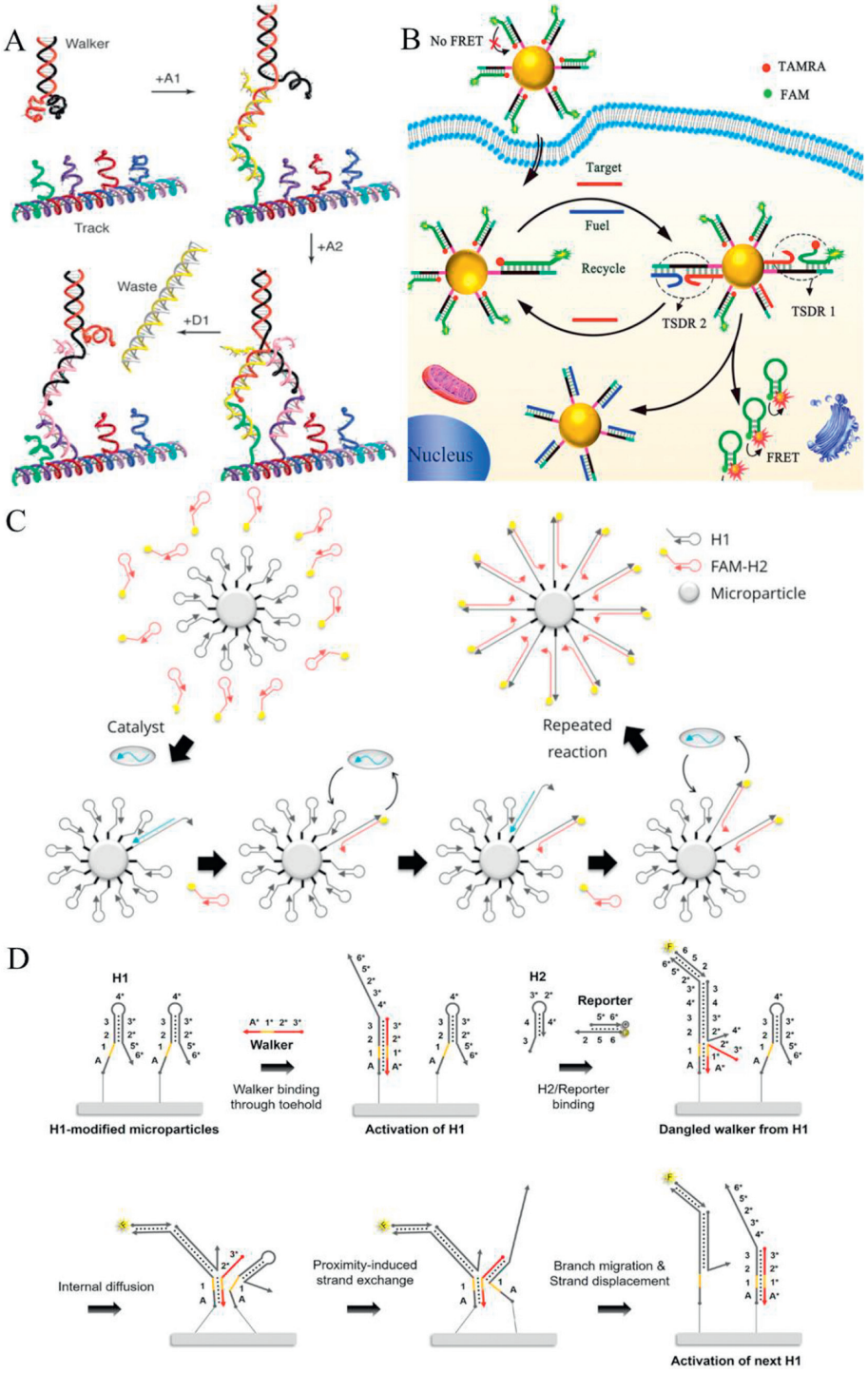

Fig. 2. Walkers propelled by strand displacement reaction. (A) A bipedal DNA walker relying on toeholds-mediated strand displacement reaction. Reprinted with permission [35]. Copyright 2004, American Chemical Society. (B) A DNA-fueled and catalytic molecule machinery for monitoring under-expressed miRNAs in living cells. Reprinted with permission [51]. Copyright 2017, American Chemical Society. (C) A stochastic DNA walker based on catalytic hairpin assembly. Reprinted with permission [12]. Copyright 2015, Springer Nature Limited. (D) A processive walker depending on a surface-immobilized proximity catalytic hairpin assembly. Reprinted with permission [52]. Copyright 2017, American Chemical Society.

a fuel strand to propel the walker operation. Ellington's group [12] reported a stochastic DNA walker that traversed a microparticle surface (Fig. 2C). Through catalytic hairpin assembly to a microparticle surface, this novel DNA walker could be triggered by a ssDNA catalyst, due to the fact that the ssDNA could open the hairpin *via* toehold-mediated strand exchange, then the opened H1 hybridized to a toehold on H2, initiating branch migration. This robust walker could realize more than 30 continuous steps movement. Subsequently, as shown in Fig. 2D, Ellington's group [52] further reported a processive walker relying on a surface-immobilized proximity catalytic hairpin assembly. H1 was modified with biotin for the link to a streptavidin-coated microparticle. Meanwhile, H2 was designed as "fuel" strand that drove the moving. Spontaneous hybridization between H1 and H2 provided the potential of the

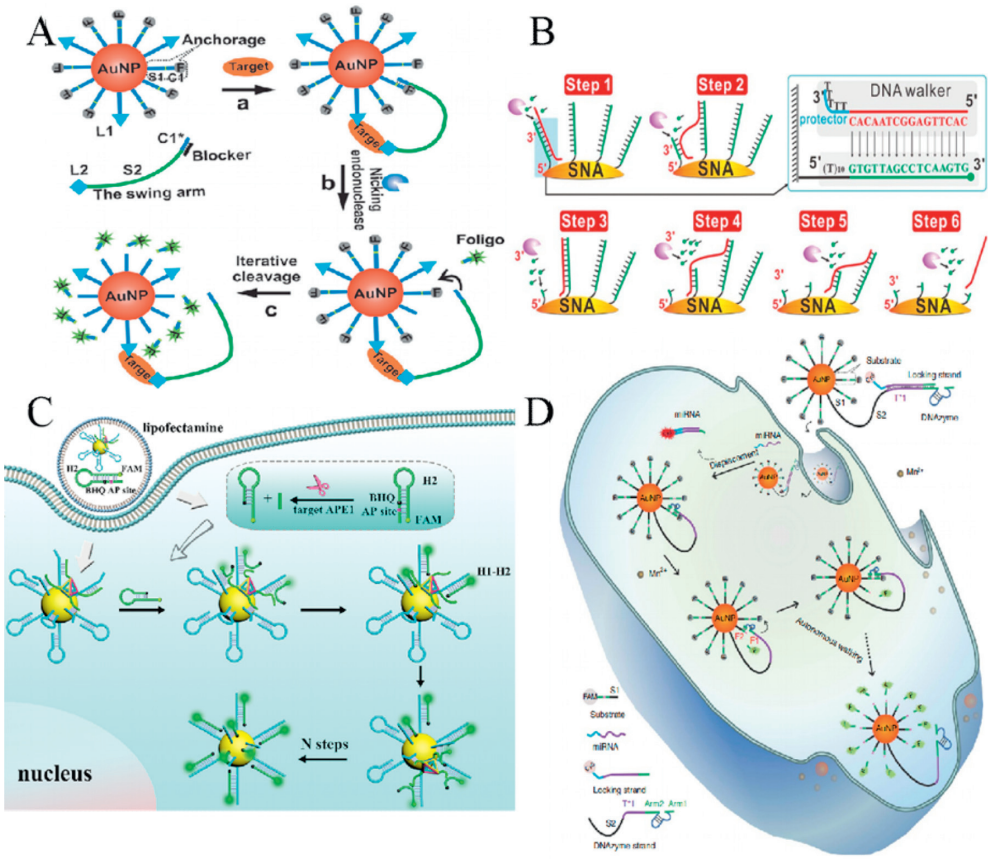

Fig. 3. DNA walker powered by different force. (A) Nicking endonuclease. Reprinted with permission [11]. Copyright 2015, Wiley-VCH Verlag GmbH. Co.KCaA.Weinheim. (B) Exo III. Reprinted with permission [41]. Copyright 2017, Wiley-VCH Verlag GmbH. Co.KCaA.Weinheim. (C) APE1. Reprinted with permission [59]. Copyright 2022, American Chemical Society. (D) DNAzyme. Reprinted with permission [60]. Copyright 2017, Springer Nature.

walker to readily search out adjacent substrates. In conclusion, different from enzymatic process, strand displacement reaction could alter the sequence length and toehold complementarity regions, holding the capability to control and engineer the intelligent DNA walker.

2.2. Protein enzyme/DNAzyme reaction

Protein enzyme and DNAzyme are ideal driving forces for the DNA walker [53–56]. With unique specificity and high efficiency, protein enzyme was widely used in biosensors, which could catalyze the breakage of polynucleotide chains by recognizing particular nucleotide sequences [53,54]. Similarly, DNAzyme is a specific DNA sequence related enzymes activity showing strong specificity and high catalytic efficiency for catalyzing the hydrolysis of particular nucleic acid substrates [55,56]. Unlike the protein enzyme denatured by environmental influence, DNAzyme is stable, programmable and modifiable. Overall, either protein enzyme or DNAzyme has its pros and cons. Nowadays, based on the enzymatic hydrolysis reaction, the protein enzyme and DNAzyme were used to be driving forces to power the DNA walker operation. In terms of operation efficiency, DNA walkers powered by the enzyme/DNAzyme presented approximately three orders of magnitude quicker than conventional walkers relying on hybridization reactions.

2.2.1. Protein enzyme reaction

Depending on the high catalytic activity, DNA walker powered by the protein enzyme hold great potential in bioanalysis. Nowadays, nicking endonuclease, exonuclease III, RNase H and apurinic/apyrimidinic endonuclease 1 (APE1), were used to be driving forces. Each protein enzyme has its own characteristics, applying in different fields. Nicking endonuclease [57], which could identify specific sequences at a strictly complementary 3′-terminus, was applied to hydrolyze specific sequence in tracks for driving the DNA walker. As shown in Fig. 3A, Le's team [11] designed the binding-induced DNA walker powered by nicking endonuclease, cleaving hundreds of oligonucleotides composed of the tracks. Based on enzymatic cleavage strategy, this DNA walker could drive the swing arm to walk progressively along the 3D-based tracks until all tracks were cleaved and the walker stopped theoretically. Even though the excellent catalytic efficiency, this walker powered by nicking endonuclease (sequence-dependent enzyme) still confronted with intrinsic limitation. Hence, it is meaningful to develop new enzyme as driving force to propel DNA walker. Compared with nicking endonuclease, exonuclease III (Exo III) as a sequence-independent enzyme is suitable for the development of a versatile sensing platform. As shown in Fig. 3B, Fan's group reported a stochastic DNA walker propelled by Exo III, autonomously walking along a spherical nucleic acid (SNA)-based 3D track *via* a burnt-bridge mechanism, realizing to monitor the operation in real time [41]. This Exo III-propelled walker exhibited high processivity due to the fact that the hydrolysis efficiency of Exo III was hundreds of times higher than those of endonucleases and DNAzymes. Despite high hydrolysis efficiency, the walker powered by Exo III was not suitable for the application in cells, as the enzyme cannot enter into cells. RNase H, playing a vital role in DNA replication, DNA repair and transcription, could specifically hydrolyze the RNA fragment in the DNA/RNA hybrid duplexes. Chu's group [58] designed a novel DNA motor achieving the in-

tracellular RNase H imaging. The walking processivity of this DNA walker propelled by RNase H surpassed that of traditional DNA walkers by three orders of magnitude. However, RNase H randomly hydrolyzed RNA sequences, limiting its analytical and diagnostic applications. In addition, APE1 as an endonuclease for maintaining genome integrity and fidelity in cells, was also developed to drive the DNA walker. Yuan's group [59] provided a novel tetrahedral DNA walker with four arms traveling efficiently on 3D-tracks, achieving APE1 image in live cells (Fig. 3C). Compared with the free bipedal DNA walker, this tetrahedral DNA walker with four arms was 4.54 times faster. However, the fixed AP cutting site also limited its application in analysis. Therefore, it is of great significance to design protein enzyme-free and simple detection probes for bioanalytical application in cells.

2.2.2. DNAzyme reaction

To break this bottleneck, DNAzymes, single-stranded DNAs identified by SELEX (systematic evolution of ligands by exponential enrichment), attracted considerable attention for biosensing and biomedical applications, due to facile synthesis, enzyme-free cleavage and high specificity. Through the cleavage of the DNA or RNA substrate, the endonuclease-mimicking DNAzyme allowed facile regulation of DNA self-assemble to realize efficient signal amplification. With the aid of metal ions, the DNAzyme could be a driving force to initiate the autonomous movement of DNA walker. As shown in Fig. 3D, Le *et al.* developed a DNAzyme motor triggered by intracellular miRNA. This DNAzyme motor is constructed on a 20 nm gold nanoparticle (AuNP), which solve the problem of enzyme entering cells [60]. This DNA motor powered by DNAzyme without the aid of DNA fuel strands and bioenzymes, showed excellent application in cells. Besides, the DNAzyme-induced DNA walker showed the potential in cell imaging, drug delivery and gene diagnosis.

3. DNA walker based on multiple dimensional tracks

DNA walkers were nanoscale- to molecular-structures consisted of self-assembly of DNA or combining DNA with other nanomaterials (*e.g.*, quantum dots, gold nanoparticles, magnetic nanoparticles, polymers), which moved at relatively high speeds (tens of nm/s) relying on the hybridization and destruction of complementary nucleic acids immobilized on a surface. DNA walkers consisted of self-assembly of DNA, taking DNA as an engineering material, have been developed to realize with controllable surface chemistry, customized sizes and shapes, and dynamic function [61,62]. Compared with self-assembly DNA walkers, DNA walkers combining DNA with other nanomaterials have more flexible application in biosensing and diagnosis, because the unique property of the nanomaterials. Either the self-assembly of DNA or combining DNA with other nanomaterials is applied to perform intricate tasks such as biosensor preparation, medicine research and water purification. So far, the DNA walkers have shown one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) tracks such as DNA nanowires, carbon tube, DNA tiles, DNA origami, AuNPs, magnetic nanoparticles.

3.1. DNA walker

As shown in Fig. 4A, the Willner's group [43] described a DNA bipedal walker consisted of four nucleic acid footholds, activated by Hg^{2+}/cysteine and H^{+}/OH^{-} triggers, striding along a 1D track. To trace moving motion, each of the footholds was labelled with fluorescence dyes, F1 (Cy5), F2 (Cy3), F3 (Cy5.5), and F4 (ROX). Meanwhile, the arm of the walker was modified with the quencher units Q (Q1, Q2) at the 5′ and 3′ ends. Under appropriate conditions, through the binding and release between the walker and the tracks, the bipedal walker could realize the mechanistic operation, and monitor the operation of the motion. Mao's group [38] developed a DNAzyme motor, transporting a nanoparticle cargo processively along a 1D RNA-coated carbon nanotube track. By rational design of the DNA walker, we explored the critical parameters which affected the translocation kinetics, including upper and lower recognition arm lengths, DNAzyme catalytic core type, and different divalent metal cations. Compared with the traditional free DNA probe limited by a homogeneous environment, the 1D DNA walker could detect the extremely low targets, by the amplification of detection signal.

3.2. DNA walker

Different from 1D tracks only possessing single direction, a 2D DNA walker possessing a planar structure has more extensible directions and rigid backbones, providing a wider range of freedom for movement of the 2D DNA walker on the DNA tracks. To control the movement, the signal molecules were labeled at the selected spots, providing a deeper understanding of intermolecular interactions.

2D DNA origami technology broke the design and construction of the 2D nanomaterial limited by the size and inerratic shape, attracting wide attention. Similar to the Seaman's DNA tiles, the DNA origami is a structure constructed by fixing crosses or junctions between DNA helices. Through many short "staple" chains for assisting the folding of a long "scaffold" DNA strand, the 2D DNA origami could present arbitrarily complex shapes such as triangle, square, rectangle and so on. As shown in Fig. 4B, Nir's team [63] designed a bipedal DNA walker which strode on a 2D DNA origami track with the help of DNA fuel and antifuel strand commands. With accurate speed and direction control, 2D DNA origami could be precise design, and this DNA walker realized 32 walking steps on a DNA origami track. This 2D DNA interface provided potential to reliably, progressively, and reproducibly control DNA walkers. Tan's group [64] applied various DNA origami to directly observe photoinduced movements of single molecules. And the stepwise of the pyrene-modified DNA walker was captured in time-lapse image. Powered by light, this DNA walker could move along a linear track on a single 2D DNA tile. Furthermore, the DNA walker also could be considered as signal amplifier. Tinnefeld's group [40] developed a nicking enzyme-initiated DNA walker moving on a rectangular DNA origami substrate, improving the detection of target molecules. Therefore, this system could be regarded as step counter or fluorescence integrator.

Despite computational, flexible and addressable functions, the DNA origami is always limited by the high cost, harsh synthesis and operating conditions. Consequently, developing other 2D materials as the tracks to improve the walking surface area and working condition are significantly important. Electrodes as prominent 2D plane have been applied to be tracks for DNA walkers. As shown in Fig. 4C, Yuan's team [65] developed a super-sensitive electrochemiluminescence (ECL) biosensor for miRNA-21 detection, relying on a DNA walking machine with triple-stranded DNA (tsDNA) nanostructures as tracks. Benefiting from the tsDNA nanostructures modified on the electrode interface averting probe entanglement and decreasing local overcrowding effects, this ECL biosensor could detect the miRNA-21 with a low detection limit of 6.67 amol/L. In addition, researchers developed some walkers based on toehold-mediated strand displacement amplification reaction. Xu's group [66] combined gold nanofilms as novel surface-enhanced Raman scattering (SERS)-electrochemistry active substrates to develop a DNA walker for dual-mode measurement of miRNA. Triggered by target miRNA and fueled by signal DNA strands labeled with rhodamine6G (R6G), this walking machine realized an enzyme-free amplification detection of miRNA with a low limit of detection.

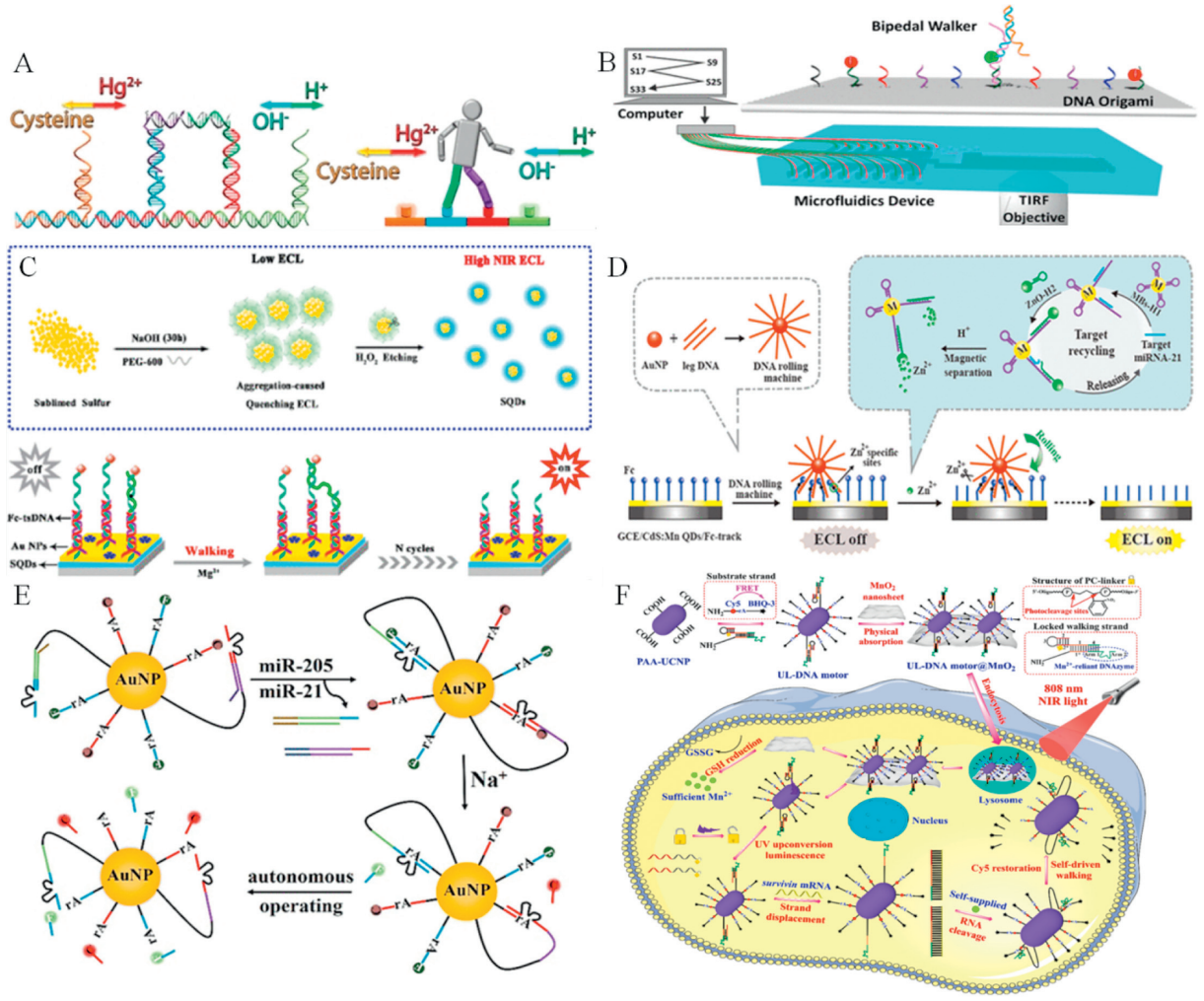

Fig. 4. Schematic illustration of (A) bipedal walker activated by Hg^{2+}/cysteine and H^+/OH^-. Reprinted with permission [43]. Copyright 2011, American Chemical Society. (B) Bipedal walker moving along the 2D DNA origami. Reprinted with permission [63]. Copyright 2017, American Chemical Society. (C) DSN enzyme-assisted target cycling amplification process. Reprinted with permission [65]. Copyright 2020, American Chemical Society. (D) The ECL biosensor based on a Zn^{2+}-driven DNA rolling machine for speedy detection of miRNA-21. Reprinted with permission [67]. Copyright 2019, American Chemical Society. (E) Intracellular Na^+-fueled DNAzyme motor differentiating cellular subtypes of non-small cell lung cancer. Reprinted with permission [55]. Copyright 2020, American Chemical Society. (F) UL-DNA motor@MnO_2 for imaging survivin mRNA. Reprinted with permission [69]. Copyright 2022, American Chemical Society.

As shown in Fig. 4D, to improve the walking efficiency, Yuan's group [67] reported an intelligent Zn^{2+}-driven DNA rolling machine constructed by numbers of leg DNA modified on Au nanoparticle, which matched with their tracks on the glassy carbon electrode. Through increasing the concentration of the leg DNA and track DNA, the walking efficiency of this walker could be improved, reducing the derailment of leg DNA. The highly speedy walking machine achieved signal amplification and provided good application in ultrasensitive strategy of miRNA detection. Therefore, the DNA walker based on 2D tracks opened a new avenue for sensitive detection of diverse biomolecules with signal amplication.

3.3. DNA walker

Up to now, the walkers that walked along 1D or 2D tracks showed excellent application in biosensor and biomedicine with signal amplification. Compared with 1D and 2D tracks, the 3D tracks constructed by the AuNPs, upconversion nanoparticles (UCNPs), microspheres, magnetic nanoparticles, and 3D origami possessing abundant specific surface area allowed the walker assembled more tracks on their surface and exhibited high walking efficiency.

Based on the origami technique, the DNA origami could be designed as 3D scale. Liu's group [68] demonstrated a plasmonic system, in which a gold nanorod could perform directional, progressive and reverse nanoscale walking on 2D or 3D DNA origami. When the walking machine carried out stepwise movements along the 3D double-layer DNA origami, the walking steps could be real-time monitored by optical spectroscopy. This walker provided another way to develop DNA walker that showed directional and progressive walking.

Besides 3D DNA origami, the AuNPs taking the advantage of unique optical property and versatile biofunctionality, have been increasingly used as good conjugated-nanomaterials for DNA walker. Tan's team [55] demonstrated a Na^+-specific DNAzyme-based DNA motor with AuNPs tracks, which could differentiate cell subtypes by simultaneously sensing miRNA-21 and miRNA-205 in live cells (Fig. 4E). Benefiting from sufficient intracellular Na^+, this miRNA-initiated Na^+-fueled DNAzyme motor avoided the adding external metallic cofactors, showing the potential application in cancer clinical diagnosis and prognosis.

UCNPs, possessing several unique features and presenting emission in the visible region upon irradiation with NIR light through a multiphoton mechanism, have been widely applied in biosens-

ing and bioimaging. Sun's group [69] reported an upconversion luminescence-initiated DNA walker, that was based on a 808 nm near-infrared light-excited low-heat upconversion nanoparticle (Fig. 4F). This light-controlled target could improve the sensitivity and specificity, due to the pre-triggering of the biosensor during the biological delivery. This DNA walker demonstrated an outstanding sensitivity with the detection limit of 0.51 pmol/L, showing the potential to distinguish single-base mismatching.

Streptavidin-coated microspheres having a spherical surface also could be regarded as nice 3D tracks. Through the streptavidin-biotin bind, the DNA strands could be modified on the surface of the streptavidin-coated microspheres. Xie's group [70] reported nicking endonuclease-powered 3D bipedal DNA walkers (BDW). The polystyrene microsphere was assembled with bipedal DNA walkers and fluorescence-labeled DNA strands. This BDW showed faster walking speed than single-legged DNA walker, and 70 min is enough for the reaction time. Meanwhile, this DNA walker have a wide linear range from 10 fmol/L to 5 nmol/L with a low detection limit of 5.2 fmol/L. Currently, Chu's group [71] also reported a DNAzyme walker, which was constructed by several DNAzyme walking strands and abundant FAM-labeled substrate strands on the surface of AuNP. This DNA walker could progressively walk along the tracks powered by DNAzyme-catalyzed substrate cleavage and realize intracellular miRNA image.

Magnetic nanoparticles were a series of nanoparticles controlled by magnetic fields, and be developed their application in medical diagnostics, imaging, drug and gene delivery. Particularly, the magnetic nanoparticles could be used in magnetic particle imaging (MPI) technology, which allowed excellent spatial and temporal resolution to enable targeted contrast enhancement and real-time assistance during medical interventions. Liu's group [72] reported a versatile single magnetic nanoparticle-confined, click chemistry actuated digital DNA walker for miRNA detection, which was fixed one DNA walking leg on a single magnetic nanoparticle. This DNA walker provided less than a 100-round walking efficiency of each swing arm, resulting in recovering sufficient fluorophores on the magnetic nanoparticle, achieving digital miRNA quantification.

4. The applications of DNA walker in biosensors

Biosensors could convert biomarker inputs into readable signal outputs such as electrochemical or fluorescence signal. Resulting from the ability of high signal amplification, the DNA walkers have tremendous potential to be sensitive biosensors. Up to now, various DNA walker-based biosensor have emerged to detect various targets such as metal ions, proteins, nucleic acids, pH, and cells.

4.1. DNA walker-based electrochemical biosensors

Electrochemical biosensors were high-profile biosensors, because of their unique features, including high detection sensitivity, excellent specificity, fast response, easy miniaturization and low-cost instruments.

As is well known, the general electrode interface could be modified with single-stranded DNA or double-stranded DNA. Xu's group [73] developed a DNA walking machine based on ECL aptasensor to quantitatively sense tumor exosomes. The DNA walking machine was constructed by the two types of DNA. One was anchor DNA as a track including nicking enzyme recognition sequences (GCTGAGG) on the loop of a hairpin strand. And another one was the swing arm-CD63 aptamer double strand. In the presence of exosomes, CD63 aptamer in the swing arm-CD63 aptamer double strand could conjugate with the CD63 protein on the exosome surface, realizing the release of CD63 aptamer and the freedom of the swing arm. Then, anchor DNA could bind with the free swing arm, generating the nicking endonuclease cleavage spot. And the movement of the DNA walking machine exhibited high controllability, providing feasible detection of exosomes and outstanding identification of different cell lines. To improve the walking efficiency, as shown in Fig. 5A, Yuan's group [74] reported an unconfined DNA walker based on a DNA three-way junction with multiple recognition regions to detect miRNA-182–5p, possessing rapid walking speed and high sensitivity. The hairpins on this DNA walker would be successively opened by target miRNA to produce an annular DNA walker, which operated on the entire scope of the electrode surface without the confine for the length of DNA walker legs, improving the walking efficiency. Wang's group [75] reported a multipedal polydopamine nanoparticles–DNA (PDANs-DNA) walker with electrochemical-driven metal–organic frameworks (MOFs) conversion-enabled biosensing platform (Fig. 5B). This walking machine has high walking efficiency due to the fact that this DNA walker with multiple legs walked fast powered by exonuclease III driving, forming DNA dendrimers through two hairpins assembly. This walker with multiple legs realized miRNA-21 detection ranging from 10 amol/L to 10 pmol/L. Besides, Lei's group [76] reported a multipedal DNA walker, which had multiple DNAzyme cores *via* rolling circle amplification. This walker was constructed by two components, the swing strand and the track. The swing strand was prepared by a padlock, an RCA primer and a block DNA as a recognition element in the DNA walker system. Meanwhile, the tracks fixed on a gold electrode, was labeled with ferrocene as signal output. Once the target DNA and an RCA kit were presented in system, RCA reaction could be activated and produce multiple DNAzyme strands for the cleavage of

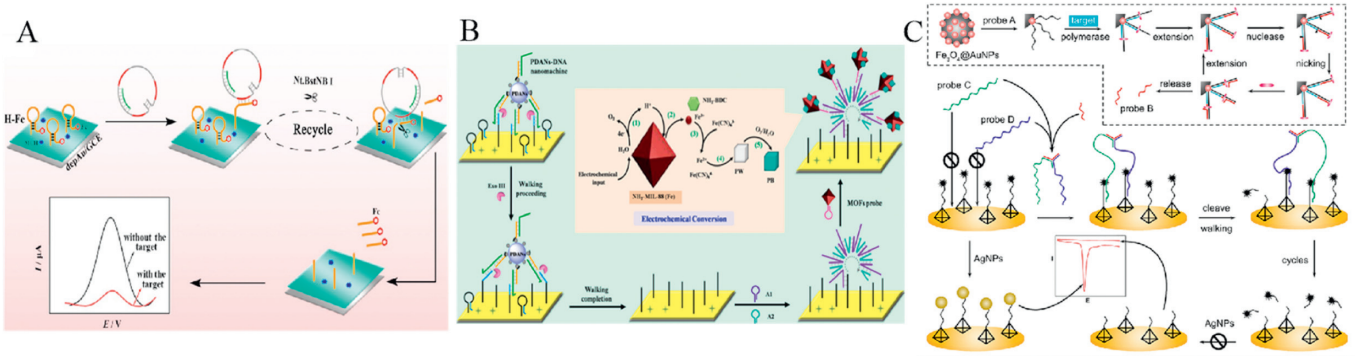

Fig. 5. Schematic illustration of (A) an as-prepared electrochemical biosensor for the detection of miRNA-182–5p. Reprinted with permission [74]. Copyright 2021, American Chemical Society. (B) Biosensor of miRNA-21 based on PDANs-DNA walker and electrochemical conversion of MOFs. Reprinted with permission [75]. Copyright 2021, American Chemical Society. (C) The bipedal DNA walker based electrochemical genosensor. Reprinted with permission [77]. Copyright 2019, American Chemical Society.

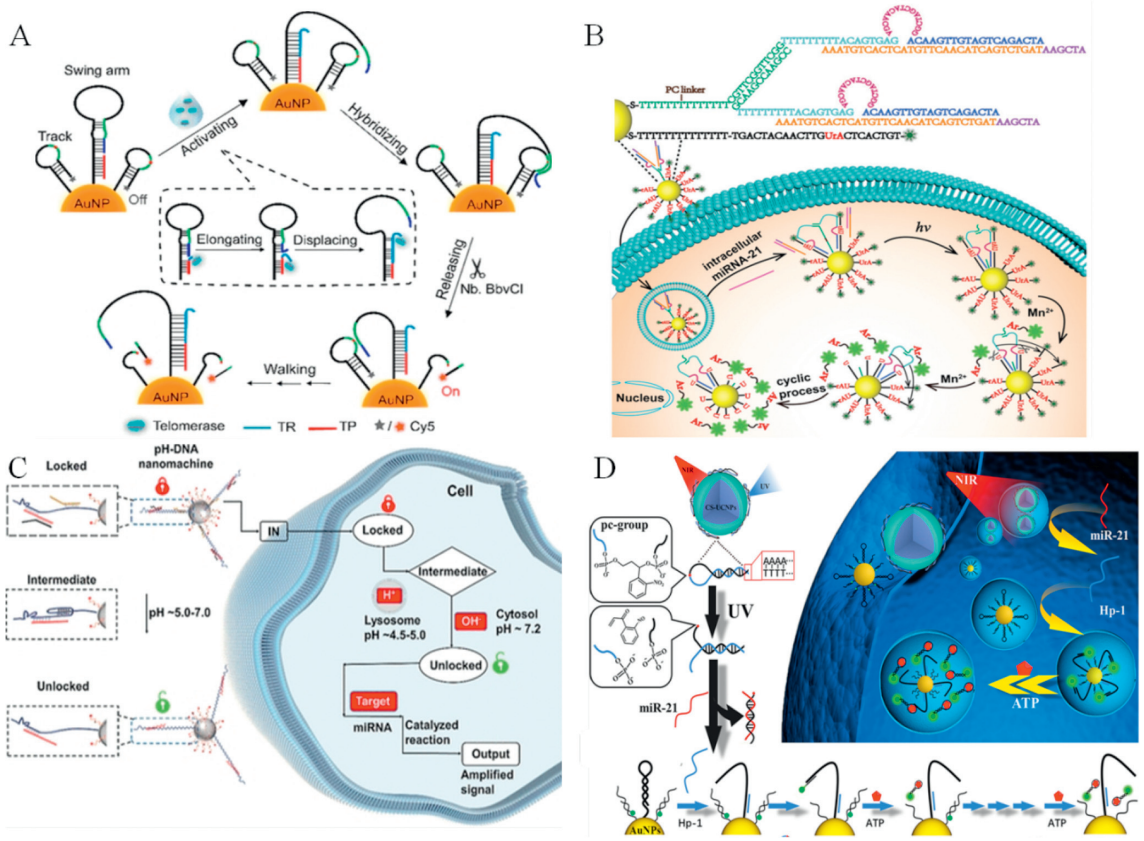

Fig. 6. Schematic illustration of (A) telomerase-triggered DNA walker for detection of telomerase activity. Reprinted with permission [80]. Copyright 2019, American Chemical Society. (B) miRNA-triggered bipedal DNA walker. Reprinted with permission [81]. Copyright 2020, RSC. (C) PRPD walker for spatially controllable imaging of intracellular miRNA through the pH sensing triple DNA. Reprinted with permission [29]. Copyright 2022, American Chemical Society. (D) Photoactivatable DNA walker system for spatiotemporally resolved miRNA sensing. Reprinted with permission [85]. Copyright 2021, American Chemical Society.

the substrates. Compared with a single and a bipedal DNA walking machine, the multipedal DNA walking machine showed a rapider cleavage speed and a lower ratio of the swing strand to the track. Despite sensitive and controllable, the above modification methods of single-stranded DNA or double-stranded DNA conjugating on the electrode suffered from local overcrowding effects and probe entanglement. To improve the steric hindrance effect, in Fig. 5C, Miao's group [77] took the tetrahedral DNA as track modified on the electrode, which could avoid spacer molecules for electrode surface modulation and enhance molecular recognition efficiency of the supported DNA track. Meanwhile, this team designed a bipedal DNA walker for electrochemical genosensing, with ultrahigh sensitivity and specificity. This proposed DNA walker provided a novel pathway to design the bipedal DNA walker with signal amplification.

4.2. DNA walker-based fluorescent biosensors

Despite high detection sensitivity, excellent specificity, fast response, easy miniaturization and low-cost instruments, the electrochemical biosensors suffering from poor cell compatibility were not appropriate to be applied *in vivo*. In consequence, it is of great significance to explore other methods used *in vivo*. Fluorescence is another signal output mode, which was widely applied to design biosensor. Due to the excellent biocompatibility, good specificity, extremely high sensitivity, and amplification capabilities, DNA walker-based fluorescent biosensors could realize *in situ* real-time biomolecules image in cells. Currently, these biosensors have been widely applied for the measurement of enzymes, pH, nucleic acids and so on [78,79].

Enzymes with catalytic effects are produced by living cells. Nowadays, some studies have reported that DNA walker-based fluorescent biosensors could achieve enzymes image in living cells. As shown in Fig. 6A, Lei's group reported a telomerase-triggered 3D DNA walker, which employed the Cy5 as the signal output [80]. The walking strand of this walker was prepared by a superhairpin structure with a bulged loop in the stem. And the dye-labeled nicking endonuclease substrates as tracks. Once the telomerase in the system, the superhairpin structure could be open, and expose the binding site of the nicking endonuclease. Then, a swing arm with the open superhairpin structure could bind with the track, achieving the fluorescence recovery by Cy5-labeled fragments released from the surface of gold nanoparticles. This proposed walker provided a new platform for the telomerase detection and broadened the design of DNA walker.

Furthermore, the DNA walker-based fluorescence biosensor was also used to apply in the detection of nucleic acids. Chu's group [71] reported a DNAzyme-based walker, initiated by intracellular miRNA-21. This DNA walker assemble DNAzyme walking strands and FAM-labeled tracks on the surface of AuNPs. Once this DNA walker entered in living cells, it could autonomously walk along the tracks fueled by DNAzyme-catalyzed substrate cleavage, and company with the FAM-labeled DNA strand release by the cleavage of substrate strand. This DNA walking machine allowed real-time operation monitor, and held the potential in the miRNA associated biological research and disease diagnostics. Furthermore, Chu's group [81] developed a photocontrolled DNA walker with bipedal DNAzyme walkers for intracellular miRNA image (Fig. 6B). Compared with a single walking machine [71] with a long operating time (180 min), this bipedal DNA walking machine enhanced the

walking machine and decreased the operating time (60 min), because of the increased number of walkers moving along the tracks. At the same time, this bipedal DNAzyme walker with a lower detection limit (3.51 pmol/L) exhibited high sensitivity and specificity. Beside the DNAzyme powered DNA walker, some researchers also developed DNA-fueled and catalytic molecule machinery. Wu's group [82] reported a self-protected DNAzyme walker capable of fully stepping on the substrate-modified AuNP for miRNAs image in live cells, exhibiting more than 8-fold enhanced catalytic activity and 5-fold enhanced serum resistance. The cleavage activity of the DNAzyme was pre-inhibited, because the catalytic core of DNAzyme walker was separated by a target binding domain. And the catalytic activity of DNAzyme walker was activated by target miRNA, resulting in the cleavage of substrates and enhancement of fluorescence signals.

Besides the above application, pH is also identified as a trigger to activate the DNA walker operate. Chao *et al.* [29] developed a pH-responsive DNA walker to perform multilayer DNA cascades, achieving calculation of intracellular biomolecules and precise sensing (Fig. 6C). Based on sequence tuning, this DNA walker could precisely respond to intracellular pH variations, because the Au nanoparticle was assembled by four-stranded DNAzyme walker precursor with a pH-responsive DNA switch, which was sensitive to pH variation of 5.0–7.0 by comformational change from linear to triplex. After the walker enter in cells, the DNA walker pass through intracellular lysosomes with acidic pH and become unlocked, enabling intracellular target miRNA image. This strategy applied an sensing-computing-output functional integration, promoting the application of programmable nanomachines in chemistry, biomedicine, and nanoengineering.

Furthermore, the DNA walker-based fluorescent biosensors were also used to detect bacteria, virus, and other micromolecule. Pei's group [61] reported stochastic DNA walkers, realizing ultrahigh-throughput bacterial detection. Through embedding intensity coded barcodes into a sequence of color-multiplexed barcodes, this multiple-step DNA walker provided a super-multiplex droplet-encoding strategy, and identified 20 distinct bacterial phenotype. By mixing encapsulated bacteria in droplets, the DNA walking machine could be triggered and the detection signals were amplified. This method provided digital and independent tuning of color and intensity, by combining with controlled ratios and amounts. And the coding capacity would grow exponentially with the increase of the number and intensity levels of the colors. This super-multiplexed and high-throughput biological assays provided enormous potential in multiple biological detection. Human immunodeficiency virus (HIV) is a virus that leads to AID by attacking the body's immune system. It is of great significance to sensitively detect HIV. Huang *et al.* [83] present an exonuclease III-propelled stochastic DNA walker for signal amplification to detect HIV DNA, achieving the detection range of 0.05–1.2 nmol/L. The AuNPs were modified with fluorescent dye-labeled hairpin DNA. The target HIV DNA can initiate the DNA walker operating powered by Exo III, generating "one-to-more" amplified fluorescence signals. This DNA walker presented good explanation of the intricate interactions among enzymes, DNA and nanoparticles, showing high promise in disease diagnosis. In addition, the DNA walker is also used to measure other micromolecules. Kanamycin as an important aminoglycoside antibiotics, has been extensively applied in the bacterial infections, pneumonia, and mastitis, because of a strong inhibiting capacity against Gram-negative bacteria. Yu's group [84] constructed an Endo IV (endonuclease IV)-powered DNA walker for ultrasensitive Kanamycin detection. The Kanamycin could bind with its aptamer, which was blocked by the complementary strand containing the Endo-IV recognition site. Through Endo IV-mediated hydrolysis of the anchorages, this DNA walker could move progressively and autonomously along 3D tracks. The cleavage fragment would be an initiator to induce the HCR reaction and Endo IV-assisted cyclic cleavage of fluorescence reporter probes. This DNA walker based on HCR amplification applied dual-amplified strategy, achieving ultrasensitive and accurate strategy to detect Kanamycin, providing effective platform for tracing amounts of analytes in molecular and bioanalysis diagnosis. Besides, the adenosine triphosphate (ATP) became an initiator to trigger the DNA walker. As shown in Fig. 6D, Xian *et al.* [85] reported a NIR-photoactivatable DNA walker powered by ATP for intracellular miRNA image with temporal and spatial resolution. In this system, UCNPs were employed to be the carriers of the DNA probe and could convert NIR to UV light. And the DNA walker could be initiated by the NIR light, fueled by intracellular ATP, achieving autonomous and progressive moving along the 3D-based track. This strategy realized intracellular miRNA image in various cell lines. Recently, tumor-derived exosomes have been attracted extensive attention, as the exosomes could be biomarkers for cancer diagnosis, due to multiple proteins on the surface of exosomes. Su's group [54] reported an exosome-responsive DNA walker, by consisting of aptamer-locked motor strands and fluorescein-labeled substrate strands. Through the recognition between the target protein on exosome and its aptamer modified on the gold nanoparticle, the DNA walker triggered by exosome could be propelled by restriction endonuclease, enabling the autonomous walking along the gold nanoparticle. Each walking step along with the cleavage of the fluorescein-labeled substrate displayed a low detection limit of 8.2 particles/μL in PBS. This proposed strategy showed great potential for developing a simple and cost-effective DNA walker for clinical diagnosis. Despite recognition of the proteins and small biomolecules, researchers also explored some DNA walkers to measure some metal ions. He's group [86] reported a programmable target-initiated DNAzyme walker with rationally designed DNAzyme that was triggered by adenosine, Ag^{+}, and target DNA sequence and so on.

4.3. Other DNA walker-related biosensors

In addition to electrochemical and fluorescent method, some DNA walkers based on other signal readout such as colorimetric patterns [87], mass spectrometric [88] and SERS [89] were presented, providing new avenues to integrate intelligent and multifunctional DNA walkers.

The DNA walkers based on the colorimetric assay were developed to detect biomolecules. As shown in Fig. 7A, Liang's group [87] described colorimetric assay based on Au-Fe_3O_4 nanocomposite, which was modified with hundreds of DNA tracks and dozens of blocked walking strands. This DNA walker could be powered by DNAzyme cleavage, and activated by the miRNA-155, producing a great deal of single-stranded fragments (displaced ssDNA). After magnetic separation, displaced ssDNA in the solution could protect AuNPs from aggregation in high-salt solutions. The miRNA-155 could be determined by UV–vis spectroscopy, and the solution color would change from grayish black to purple. This facile, sensitive and specific colorimetric assay achieved the miRNA-155 detection in real human serum samples without complicated sample pretreatment.

Mass spectrometry as a superior accurate analytic technique for ratiometric measurements, showing a mass spectrum that plots the mass-to-charge (m/z) ratio of compounds in a mixture, has been extensively applied in metal stable isotope tagging. As shown in Fig. 7B, Liu *et al.* [88] developed a ratiometric DNA walker based on the elemental isotope ratio measurement for the detection of target DNA in human serum and cell lysates. Through a burnt-bridge mechanism, this Endo IV powered DNA walker could convert and amplify biological signals into mass spectrometric ratio signals ($^{197}Au/^{115}In$). This target-triggered DNA walker could au-

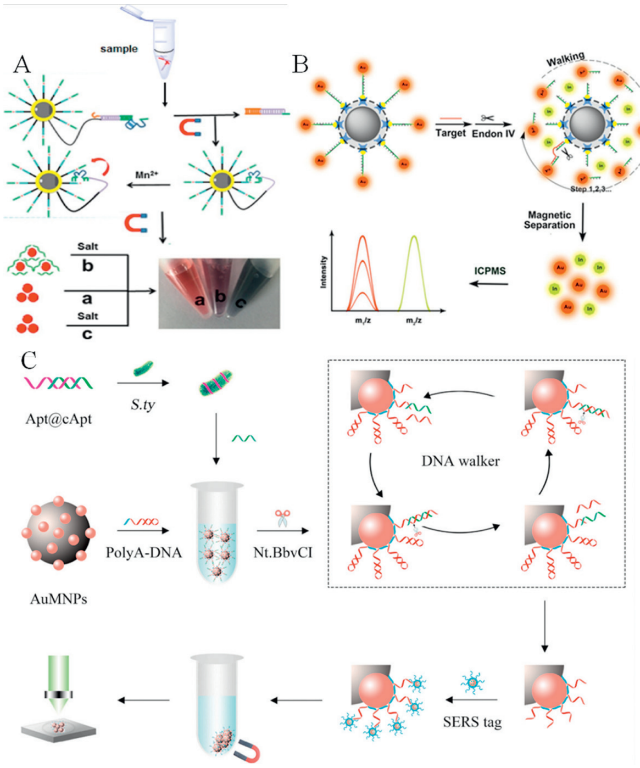

Fig. 7. Schematic illustration of DNA walker based on (A) colorimetric strategy. Reprinted with permission [87]. Copyright 2020, Elsevier B.V. All rights reserved. (B) Mass spectrometry. Reprinted with permission [88]. Copyright 2019, Wiley-VCH Verlag GmbH. Co.KCaA.Weinheim. (C) SERS strategy. Reprinted with permission [89]. Copyright 2021, Elsevier B.V.

tonomously walk along the 3D-AuNPs. Then magnetic microparticle was used to separate the AuNPs after hybridization of DNA tracks and the target DNA. This strategy successfully converted the output signal to a mass spectrometric signal for ratio analysis. This proposed DNA walker provided a versatile platform for small biomolecules and proteins measure.

Additionally, SERS possesses the advantages of extremely narrow peak width, high sensitivity multivariate detection ability and wide dynamic range (Fig. 7C). Recently, Duan's group [89] developed a 3D DNA walker based on the SERS strategy for quantitative analysis of Salmonella typhimurium (S.ty). This DNA walker taking AuNPs modified magnetic nanoparticles as 3D walking surface, could be triggered by the complementary DNA of S.ty-recognizing aptamer replaced from the double-stranded DNA (ds-DNA) of Apt@cApt reacting with S.ty. Under the driving force of nicking endonuclease, this system could induce the SERS signal. This DNA walker showed highly selective and sensitive method for the S.ty detection, achieving the signal amplification, presenting new methods for SERS assay of biomaterials.

5. Conclusions and future perspectives

In this review, we have comprehensively summarized the development of the DNA walkers, and their application in biosensor and bioimaging. Based on precise programmability and addressability, DNA walker with amplification strategies show high sensitivity, selectivity and motility for biomolecules, such as ions, nucleic acids, proteins, and bacteria, *etc*. Meanwhile, the detection limit of the most DNA walkers was relatively low, due to amplification capability, and high sensitivity for the target. However, the DNA walkers always remain some challenges for the researchers to develop more intelligent walker to complete complex tasks. First, it is of great significance to enhance the efficiency of DNA walker. Limiting by the ratio of the walking strands to the tracks, categories of driving forces and migration rate, the efficiency of DNA walker was relatively low. Currently, the researchers applied strategies to increase the ratio of the walking strands to the tracks, or change the ways of migration, developing rotating or rolling machine, and bipedal or multiple walker to improve the contact chance between the walking strands and the tracks, and reduce the interference from complex solution. Additionally, the design of the DNA walker including the structure of the DNA walker, the track, and the cleavage site also affect the efficiency. Second, the surface regulations of DNA walkers were also critical to improve walking speed, accuracy, efficiency, and biological applications. Generally, the sequences, length, and density of DNA probes affect spatial position resistance of the DNA assemblying on the vector surface, favorable to improve high anti-interference capacities of the DNA walker-based nanosystems, which were beneficial to the development of novel and highly efficient DNA walker. In addition, the type and size of DNA vectors also affect the DNA assemblying on the surface, thereby influence the operating efficiency of the DNA walker.

The DNA walkers were also identified as amplification strategies with great sensitivity and specificity. Due to the addition of fuel strands, protein enzyme and DNAzyme, the DNA walker strand could cleave the tracks circularly, along with the signal amplification. Once the target in the system, the DNA walker could be triggered and operate autonomously and progressively along the tracks. Therefore, the DNA walkers have great potential to prepare intelligent walker.

In summary, based on the programmability and predictability of DNA structures, the DNA walkers were designed to execute various tasks, and explored the potential application into different fields, such as biosensor, bioimaging, smart drug delivery vehicles. In brief, the development of the DNA walker could be summarized as follows: (1) to create a variety of walking mode for improving the operating efficiency of DNA walkers; (2) to ingeniously design the DNA structure for performing a diverse range of tasks such as clinical diagnosis, environmental monitor and food safety analysis; (3) to develop highly sensitive and selective sensing platforms by expanding various signal output forms, *e.g.*, electric, fluorescence, SERS. Generally, it is a long journey and arduous challenge to develop more efficient, fast and sensitive DNA walker.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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