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Review

Nucleic acid-based fluorescent methods for the determination of DNA repair enzyme activities: A review

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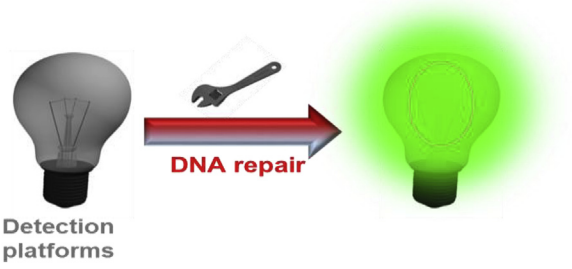
HIGHLIGHTS

- Nucleic acid-based fluorescent methods for assaying DNA repair enzymes, are summarized.
- Novel assays which utilize functional nucleic acids, are discussed.
- Ultra-sensitive strategies that rely on isothermal nucleic acid amplification, are discussed.
- Future directions in the area of fluorescent enzyme assays are suggested.

GRAPHICAL ABSTRACT



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ABSTRACT

DNA repair pathways are closely associated with the maintenance of genomic integrity, disease outbreak, and the development of therapeutics. Owing to these significances, novel analytical methods for enzymes that are involved in the DNA repair pathways have been actively investigated. This review focuses on discussions on nucleic acid-based methods, especially those based on the fluorescence, for the determination of DNA repair enzymes. Furthermore, this review not only provides meaningful insights in creating ingenious fluorescent detection methods but it also suggests future directions that will be followed in developing new analytical technologies for the DNA repair enzymes.

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1. Introduction

DNA that encodes genetic information, constantly experiences damage caused by endogenous and exogenous factors such as reactive oxygen species, ultraviolet light and a variety of chemicals [1]. Every day, tens of thousands of lesions take place in human DNA [2], which either induces the formation of DNA mutations through several cycles of DNA replication, or interrupts DNA replication and other cellular processes [3–5]. If DNA lesions remain unrepaired, they lead to lethal consequences such as premature aging, developmental disorders, and cancers [6–8]. For example, the presence of 8-oxoguanine (8OG, Table S1), which mispairs with adenine during replication, induces a G:C→T:A transversion mutation, the second most common somatic mutation found in cancers [9–11]. Fortunately, cells have evolved elaborate defense systems for monitoring and repairing genomic errors [12]. A representative example is the base excision repair (BER) pathway that is recruited to ameliorate base lesions. Various enzymes, including DNA glycosylases, AP endonucleases, end-processing enzymes, DNA polymerases and DNA ligases, participate in this pathway to maintain genomic integrity [13]. Importantly, enzymes involved in DNA repair pathways have been considered as attractive targets for the treatment of diseases such as cancers and AIDS. Accordingly, their inhibitors have been extensively investigated as potential drugs [14–17].

Until now, a wide range of methods have been created for assaying DNA repair enzymes. For example, gel electrophoresis together with radioactive labeling has been the 'gold standard' method [18,19]. Other methods based on high performance liquid chromatography (HPLC) [20], enzyme-linked immunosorbent assay (ELISA) [21], capillary electrophoresis [22], comet assay [23], and western blot with immunohistochemistry [24,25] have also been developed. However, these techniques are complex, labor/time-intensive, and costly, all of which hamper their practical utilization. This consequently stimulated the development of new methods that overcome the drawbacks of the previous assays.

Special attention has been given to fluorescence-based strategies which are advantageous in high sensitivity, simplicity and robustness, resulting in the development of efficient protocols that operate in a simple "mix-and-detect" manner [26]. In most of these fluorescence-based assays, nucleic acids have been utilized as key sensing elements due to their intrinsic beneficial features including ease of chemical synthesis/modification, high robustness and unique structures such as single/double-strand, G-quadruplex, and i-motif [27,28]. Notably, fluorescent nanomaterials, generated from nucleic acids in the form of silver nanoclusters (AgNCs) and copper nanoparticles (CuNPs), have been found to have outstanding photophysical properties, low toxicities, and high biocompatibilities [29–34]. Owing to these features and the fact that their fluorescence properties are responsive to sequence and conformational changes of nucleic acids, these nanomaterials have been used in the design of new fluorescence-based enzyme assays [29–31,35–38].

In addition, nucleic acids that are capable of catalyzing biochemical reactions, termed deoxyribozymes (DNAzymes), have been employed as a novel detection unit [39–42]. Importantly, the use of DNAzymes that have high turnover rates enables sensitive determination of enzyme activities [43–46]. Moreover, DNA aptamers, nucleic acids that possess strong affinities toward specific target molecules, have emerged as promising alternatives to antibodies for use as affinity receptors and been used in the design of fluorescence-based enzyme assays [47–50]. Lastly, nucleic acids have been linked to isothermal nucleic acid amplification methods that operate at a constant temperature without the requirement of expensive instrumentation. A number of novel strategies relying on RCA, LAMP, EXPAR, CHA and HCR, have been attempted for the ultrasensitive determination of DNA repair enzyme activities [51–55].

In this review, nucleic acid-based fluorescent strategies to analyze DNA repair enzyme activities are discussed according to key components of individual detection platforms. We believe that this review will provide general understanding on the development of detection systems along with interesting techniques that have been achieved so far, and also suggest a clue in designing novel detection strategies for DNA repair enzymes.

2. Determination of DNA repair enzyme activities

Owing to their increasing importance, DNA repair enzymes have elicited a tremendous number of studies aimed at developing novel and efficient detection methods [51]. In the following section, major methods for assaying DNA repair enzymes are classified based on their key components and discussed in detail (Table 1). The repair enzymes covered in this review, are listed in Table 2 along with their activities.

2.1. Molecular beacons

This section focuses on detection strategies that utilize molecular beacons (MB), hairpin structured molecules modified with a fluorophore and quencher at both ends. The MB developed by Tyagi et al. was first applied to the detection of target DNAs based on the working principle that the initially quenched fluorescence of MB is recovered by a conformational change induced by the presence of target DNA [56–58]. In follow-up studies aimed at devising a system for determination of DNA repair enzyme activities, the MB was engineered to serve as a substrate for the DNA repair enzyme. For instance, hOGG1, a BER enzyme specific to 8OG, removes 8OG

Table 1
Comparison of the analytical methods for the DNA repair enzymes discussed in this review.

Target		Key component	Element	Assay time (hr)	Detection limit (U/mL)	Limitations	Reference
Glycosylases	UDG	Nanobeacon	GO	2.5	0.0008	- Labeling with fluorophore - Use of Endo IV	[74]
			ScGFP	0.5	0.0015	- Preparation of ScGFP - Use of Endo IV - Turn-off	[81]
		FNA	2A	1	0.02	–	[84]
		DNA conformation-specific fluorescent dye	Ir complex	0.5	0.02	Preparation of inorganic signaling molecule	[99]
			ATMND	0.2	0.0008	–	[108]
		DNA-templated fluorescent nanomaterials	CuNPs	1.7	0.0005	Turn-off	[113]
		DNAzyme	DNAzyme	Real-time	0.0034	Labeling with fluorophore and quencher	[44]
		DNA aptamer	DNA polymerase-specific aptamer	2.2	0.024	- Labeling with fluorophore and quencher - Use of DNA polymerase	[142]
		Isothermal nucleic acid amplification	EXPAR	2	0.0001	- Labeling with fluorophore and quencher - Use of several enzymes - Complex probe design	[52]
			NEAR	1.2	0.003	- Use of several enzymes - Turn-off	[148]
			RCA	5.5	0.00017	- Use of several enzymes - Time-consuming	[153]
			LAMP	2	0.00068	- Use of several enzymes - Complex probe design	[55]
			CHA	2.75	0.00044	–	[163]
			HCR	2.2	0.00006	Labeling with fluorophore	[169]
			2-Layered HCR	1	0.00011	- Labeling with fluorophores - Complex probe design	[172]
	hOGG1	MB	MB	–	–	Labeling with fluorophore and quencher	[59]
	Fpg	FNA	P	1.5	1.25	–	[85]
Endonucleases	EcoRI	DNA conformation-specific fluorescent dye	SGI	Real-time	8	Turn-off	[97]
		Isothermal nucleic acid amplification	HCR	5	0.41	–	[170]
Exonucleases	Exo I	DNA aptamer	DNA polymerase-specific aptamer	3	0.22	- Labeling with fluorophore and quencher - Use of DNA polymerase	[142]
	Exo III	MB	Stacked G quencher	Real-time	0.04	Labeling with fluorophore	[60]
		DNA conformation-specific fluorescent dye	SGI	1.2	0.7	Turn-off	[96]
			CV	Real-time	5	–	[98]
Polymerases	KF	DNA conformation-specific fluorescent dye	ThT	Real-time	0.05	–	[101]
Polynucleotide kinases	T4 PNK	Nanobeacon	CoOOH	0.5	0.01	- Labeling with fluorophore - Turn-off	[71]
			WS_2	0.5	0.01	- Labeling with fluorophore - Turn-off	[77]
		DNA-templated fluorescent nanomaterials	CuNPs	2	0.05	Use of several enzymes	[116]
		Isothermal nucleic acid amplification	CHA	1	0.000093	Labeling with Py	[159]
			HCR	4.6	0.000337	–	[171]
Ligases	*E. coli* DNA ligase	MB	MB	Real-time	0.0004	Labeling with fluorophore and quencher	[63]
Single-strand specific nucleases	S1 nuclease	DNA-templated fluorescent nanomaterials	AgNCs	2	0.01	–	[115]
			CuNPs	2	0.3	Turn-off	[112]
RNases	RNase H	MB	Py	0.25	5	Labeling with Py	[68]
		Nanobeacon	AuNPs	1	0.0043	- Labeling with fluorophore - Modification of AuNPs	[76]
			GO	0.7	0.005	Labeling with fluorophore	[75]
		DNA conformation-specific fluorescent dye	NMM	0.3	0.2	–	[100]
		DNAzyme	DNAzyme	0.7	0.01	Labeling with fluorophore and quencher	[43]
		Isothermal nucleic acid amplification	RCA	Real-time	0.019	Use of several enzymes	[154]
			CHA	3	0.037	–	[162]

Table 2
The DNA repair enzymes discussed in this review.

DNA repair enzyme	Role	Corresponding enzyme	Specific function
Glycosylases	Recognize and excise damaged nucleobases by cleaving the N-glycosidic bond between nucleobase and deoxyribose, leaving the AP site or cleaving the DNA backbone at the resulting AP site in BER.	UDG	Releases uracil (U) nucleobases from U-containing DNA, leaving the AP site.
		hOGG1	Releases 8OG nucleobases from dsDNA, and cleaves the 3′ to the resulting AP site, leaving the nick site.
		Fpg	Releases 8OG nucleobases from dsDNA, and cleaves both 3′ and 5′ to the resulting AP site, leaving the gap site.
Endonucleases	Recognize and cleave the DNA backbone at the AP site, or damaged DNA patches in BER and nucleotide excision repair (NER).	Endo IV	Cleaves the 5′ to the AP site, leaving the nick site.
		EcoRI	Recognizes the specific recognition site and cleaves as shown below, where '/' denotes the nick site. 5′-G/AATTC-3′ 3′-CTTAA/G-5′
Exonucleases	Cleave damaged DNAs from their ends, which is essential in mismatch repair (MMR) and homologous recombination (HR).	Exo I	Digests the ssDNA from the 3′-end.
		Exo III	Digests the duplex from the 3′-ends.
Polymerases	Replace excised nucleotides with appropriate ones in BER, NER, MMR, and HR.	KF	Executes the synthesis of DNA with the strand displacement activity.
Polynucleotide kinases	Transfer the γ-phosphate from ATP to the 5′-hydroxyl group of DNA, which aids the ligase to complete the DNA repair process.	T4 PNK	Transfer the γ-phosphate from ATP to the 5′-hydroxyl group of DNA.
Ligases	Link the 3′-hydroxyl and 5′-phosphate groups by forming a phosphodiester bond between DNAs, which is required in the most of repair processes including BER, NER, MMR, and so on.	*E. coli* DNA ligase	Catalyzes the formation of the phosphodiester bond between juxtaposed 5′-phosphate and 3′-hydroxyl ends in duplex DNA.
		T4 DNA ligase	Is similar with that of *E.coli* DNA ligase, but can be applied to the duplex RNA and DNA/RNA hybrid.
Single-strand specific nucleases	Degrade single-stranded (ss) nucleic acids, which is involved in BER, NER, and MMR.	S1 nuclease	Degrades ss nucleic acids with the preference on DNA than RNA.
RNases	Prevent the misincorporation of ribonucleoside monophosphates into DNA during the replication by degrading RNA.	RNase H	Hydrolyzes the phosphodiester bonds of RNA hybridized to DNA.

positioned in the stem of a MB and excises the resulting abasic (AP) site, leading to spatial separation of a fluorophore (F) from a quencher (Q) with a concomitant fluorescence increase (Fig. 1(a)) [59]. A sensing system for Exo III activity was also developed using a similar MB-based strategy, in which stacked guanines (Gs) function as a fluorescence quencher (Fig. 1(b)) [60–62]. Another example focused on analysis of DNA ligase activity. In this method, ligation of separated probes on the loop of an MB occurs to stretch the hairpin into a linear structure, thereby separating the F and Q groups and bringing about a significant fluorescence enhancement (Fig. 1(c)) [63].

In addition, pyrene (Py), the excimer-forming aromatic hydrocarbon, which has served as a promising signaling molecule in bioassays [64–67], was employed instead of a fluorophore and quencher pair in a MB designed to determine RNase H activity [68]. In the presence of RNA, a hybridization complex is formed, causing

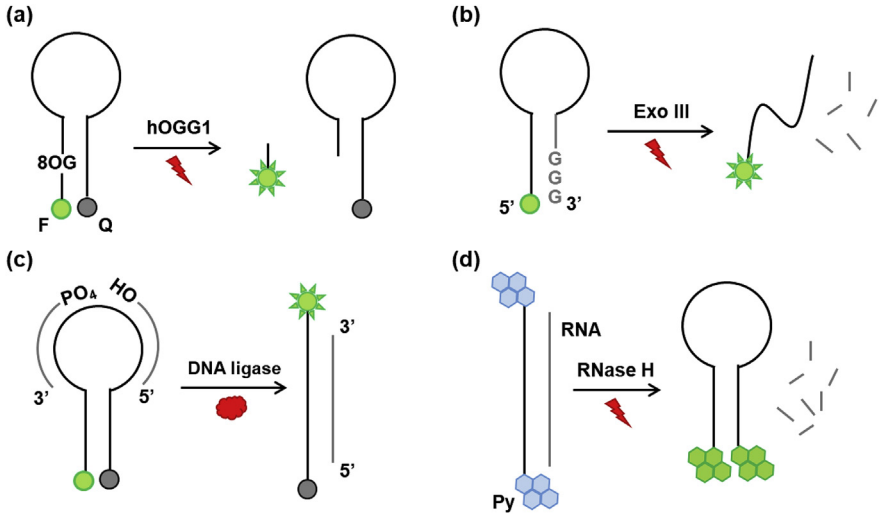

Fig. 1. MB-based fluorescent methods for detection of DNA repair enzymes. (a) hOGG1 activity assay using MB modified with F and Q [59]. (b) Exo III activity assay using MB modified with F and stacked Gs [60]. (c) DNA ligase activity assay using MB modified with F and Q, where the separated ligation probes are hybridized [63]. (d) RNase H activity assay using MB modified with Pys at both ends [68].

the Pys to be remotely located. However, the Py groups move into close proximity when RNA in the RNA/DNA hybrid is specifically degraded by RNase H. This close location of the Pys consequently leads to formation of an excimer that emits light at longer wavelengths than monomeric Py does (Fig. 1(d)) [68].

2.2. Nanobeacons

Novel nanomaterials, such as gold nanoparticles (AuNPs), graphene oxide (GO), cobalt oxyhydroxide (CoOOH) and tungsten disulfide (WS_2), have been exploited as compelling alternatives to organic fluorescence quenchers in newly developed strategies, while keeping the simplicity of probe design in the MB-based methods, which are termed nanobeacons [69–73]. In these systems, DNA repair enzymes, including RNase H and UDG, promote release of a F from AuNPs or GO by cutting off a F-labeled strand, which results in an increase in the fluorescence intensity (Fig. 2(a~c)) [74–76]. In a similar manner, an activity of T4 PNK is determined in combination with nanomaterials such as CoOOH or WS_2. The 5′-end of DNA (gray) is phosphorylated, followed by the degradation by lambda exonuclease (λ Exo). The remaining F-labeled ssDNA (black) is then adsorbed onto the nanomaterials with the reduction of its fluorescence (Fig. 2(d)) [71,77].

Another interesting strategy for detecting DNA repair enzymes relies on a supercharged protein exhibiting an unusually large net charge [78–80]. In this research, a positively supercharged green fluorescent protein (ScGFP) forms a polyionic complex with the negatively charged DNA, which prevents its adsorption onto GO and subsequent fluorescence quenching [81]. Importantly, Wang et al. found that this process is dependent on the length of DNA, only occurring with the lengths over 5 nt (nucleotide) [81]. Based on this novel finding, the UDG activity is determined in a process that UDG and Endo IV cleave DNA into shorter DNAs whose lengths are too short to block the adsorption and fluorescence quenching of ScGFP by GO (Fig. 2(e)) [81]. Overall, these nanobeacon-based platforms with the remarkable fluorescence quenching abilities exhibit better sensitivities than the counterparts that require the labeling with organic quenchers [74,75,82,83]. However, the nanomaterials themselves can inhibit enzyme activities, and thus their separate addition is required after the completion of enzymatic reaction. The “mix-and-read” format that enables the real-time monitoring of enzyme activity is not achieved, which has remained as a critical drawback.

2.3. Fluorescent nucleobase analogs

New platforms for detecting DNA repair enzymes have been designed by incorporating a fluorescent nucleobase analog (FNA), which do not require external labeling by both fluorophore and quencher [84–86]. The FNA exhibits conformation-dependent fluorescence properties while retaining the ability to participate in Watson-Crick base pairing. Specifically, its fluorescence of duplexes is significantly quenched as a consequence of both stacking interactions and collisions with surrounding nucleobases, while being enhanced when the duplex is dissociated into ssDNAs [86–89]. Taking advantage of this unique fluorescence property of FNAs, the activity of Fpg involved in DNA repair, was analyzed in a simple and cost-effective manner, where pyrrolo-dC (P), a fluorescent analog of cytosine, was employed (Fig. 3(a)) [85]. Another simple, yet efficient strategy was developed to assay UDG activity. This approach relies on a novel finding that the fluorescence of 2-aminopurine (2A), an emitting analog of adenine, which is site-specifically incorporated into a G-rich sequence, enormously increases upon formation of G-quadruplex because pi-pi stacking and electron transfer quenching of 2A by surrounding bases is suppressed [90–92]. In this system, the fluorescence of 2A is significantly enhanced only in the presence of UDG, which catalyzes the removal of U nucleobases and promotes formation of a G-quadruplex (Fig. 3(b)) [84]. This example suggests that the rational probe designs considering interactions between FNA and surrounding bases would pave the way for the development of other DNA repair enzyme assays.

2.4. DNA conformation-specific fluorescent dyes

Label-free strategies for assessing DNA repair enzyme activities, which utilize DNA conformation-specific fluorescent dyes, have also been intensively investigated. The first system employs the

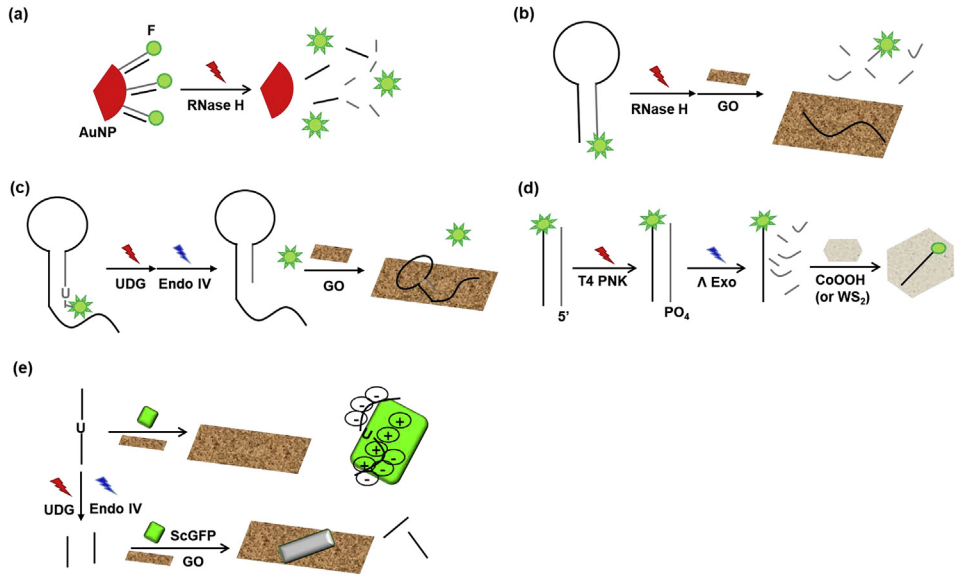

Fig. 2. Fluorescent methods for detection of DNA repair enzymes, which utilize nanobeacons. (a) RNase H activity assay using AuNPs as a fluorescence quencher [76]. (b) RNase H activity assay using GO as a fluorescence quencher [75]. (c) UDG activity assay using GO as a fluorescence quencher [74]. (d) T4 PNK activity assay using CoOOH (or WS_2) as a fluorescence quencher [71,77]. (e) UDG activity assay using ScGFP as a fluorescence reporter and GO as a fluorescence quencher [81].

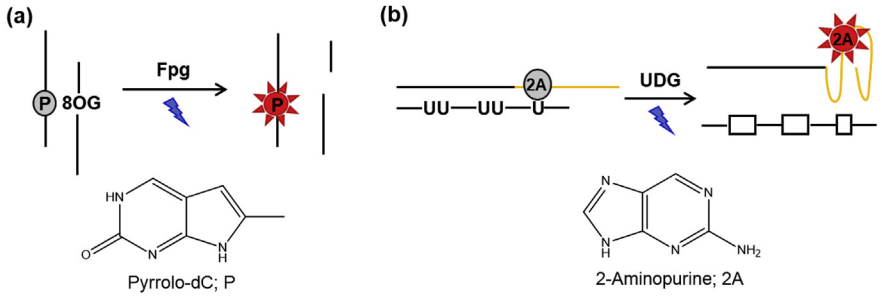

Fig. 3. FNA-based fluorescent methods for detection of DNA repair enzymes. (a) Fpg activity assay using pyrrolo-dC (P) as a FNA [85]. The chemical structure of P is shown below. (b) UDG activity assay using 2-aminopurine (2A) as a FNA [84]. The chemical structure of 2A is shown below.

fluorescent dye, SYBR green I (SGI), which exhibits enhanced fluorescence after binding to the DNA duplexes [93–95]. In this system, DNA repair enzymes such as EcoRI and Exo III cleave DNA duplex into ssDNAs with subsequent release of SGI and production of a diminished fluorescence signal (Fig. 4(a)) [96,97]. Another system relies on the use of G-quadruplex specific fluorescent dyes, such as iridium (III) complexes, Tb^{3+}, N-methyl mesoporphyrin IX (NMM), crystal violet (CV) and thioflavin T (ThT), which display significantly enhanced fluorescence after binding to the G-quadruplex structure [98–104]. In this case, a DNA substrate that initially cages the G-rich sequence is broken down by the target enzymes to produce the G-quadruplex, which then interacts with the G-quadruplex-specific fluorescent dye [98–101]. This strategy has been employed to develop methods to detect various DNA repair enzymes including UDG, RNase H, Exo III, KF, and T4 PNK (Fig. 4(b ~ e)) [98–101,105–107]. It should be noted that these systems are capable of monitoring the enzyme activities in real-time since their fluorescent dyes do not affect, but rapidly respond to enzymatic reactions. However, they are not as sensitive as the methods utilizing other key components such as DNA-templated fluorescent nanomaterials or isothermal nucleic acid amplification, which might be due to the inefficient fluorescence properties of dyes or the absence of amplification steps (Table 1).

Another sensor was devised by Tao et al. In this sensor, the AP site-specific fluorescent dye, 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND), is used to determine UDG activity. Specifically, ATMND initially binds to a non-base spacer (dSpacer; S) opposite to the U nucleobase in duplex DNA through hydrogen bonding and pi-pi stacking interactions, a process that results in quenching of the fluorescence of the dye. In the presence of UDG, which catalyzes removal of the U nucleobase, ATMND is released from S in the duplex DNA, giving rise to an enhanced fluorescence signal. With this simple detection principle, the UDG activity was sensitively determined (Fig. 4(f) and Table 1) [108].

2.5. DNA-templated fluorescent nanomaterials

Since their development, DNA-templated fluorescent nanomaterials such as metal nanoclusters and nanoparticles have emerged as promising alternatives to conventional organic

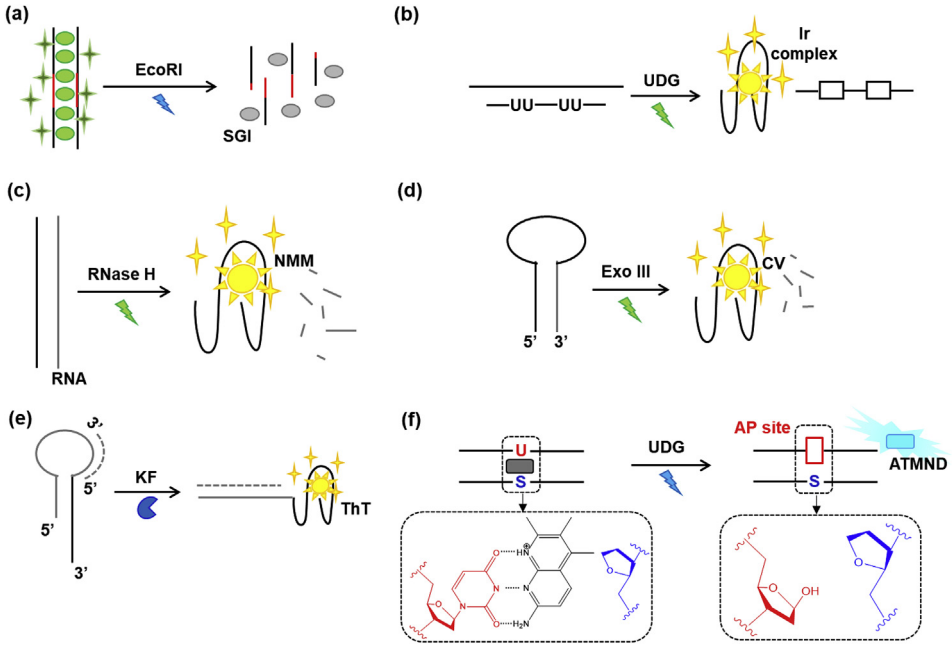

Fig. 4. Fluorescent methods for detection of DNA repair enzymes, which utilize conformation-specific fluorescent dyes. (a) EcoRI activity assay using a duplex-specific fluorescent dye, SGI [97]. (b) UDG activity assay using a G-quadruplex-specific fluorescent dye, Ir complex [99]. (c) RNase H activity assay using a G-quadruplex-specific fluorescent dye, NMM [100]. (d) Exo III activity assay using a G-quadruplex-specific fluorescent dye, CV [98]. (e) KF activity assay using a G-quadruplex-specific fluorescent dye, ThT [101]. (f) UDG activity assay using an AP site-specific fluorescent dye, ATMND [108]. 'S' in blue denotes a non-base spacer (dSpacer). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fluorophores owing to their unique properties including high fluorescence quantum yield, photostability and facile synthesis [29,30,32,33]. In particular, because their fluorescence is sensitive to alterations in the DNA template or its surroundings, these nanomaterials are useful for monitoring enzyme activities [109–113]. Thus, several convenient, fluorescent assays utilizing DNA-templated nanomaterials, have been developed to determine DNA repair enzyme activities.

The first sensing system employs DNA-templated silver nanoclusters (DNA-AgNCs), whose fluorescence is significantly enhanced when being in close proximity to the G-rich sequence [114]. As illustrated in Fig. 5(a), S1 nuclease that degrades ssDNA substrate (gray) enables G-DNA to hybridize with DNA-AgNCs. As a consequence, the G-rich sequence (green) of G-DNA gets closer to the AgNCs, resulting in significantly enhanced fluorescent emission of AgNCs [115]. The second example is comprised of DNA-templated fluorescent copper nanoparticles (CuNPs) [29,30]. In this method, dsDNA, which contains the U nucleobases, serves as a template for the formation of CuNPs. However, in the presence of UDG, which removes the U nucleobases, the duplex probe is transformed into ssDNAs, which then does not participate in the production of fluorescent CuNPs (Fig. 5(b)) [113]. The same principle was also applied to develop a sensor for determining S1 nuclease activity (Fig. 5(c)) [112]. In addition, another strategy utilizing the DNA-templated fluorescent CuNPs was devised for the sensitive detection of T4 PNK activity [116]. In this system, the presence of T4 PNK aids ligation of a dumbbell probe and the ligated probe is protected against exonuclease-mediated digestion, enabling formation of the fluorescent CuNPs. In contrast, in the absence of T4 PNK, the dumbbell probe is not ligated and is subsequently digested by the exonucleases. As a result, CuNPs are not formed, resulting in a near zero background signal, which enables sensitive determination of T4 PNK activity (Fig. 5(d)) [116]. Although promising, these systems are vulnerable to the reaction conditions, especially buffer composition, because the fluorescence efficiencies of nanomaterials can be compromised by the high salt concentration that is essential for target enzyme activities [114,117,118].

2.6. DNAzymes

DNAzymes are ssDNA that is capable of catalyzing various biochemical reactions [119–123]. DNAzymes have several interesting properties including facile preparation, high stability and ease of modification. In particular, because their activity depends on cofactors, DNAzymes have been utilized to detect various metal ions and amino acids [124–128]. Furthermore, they have been linked to additional target recognition units, such as complementary DNAs and aptamers, to create systems for detecting various biological substances including nucleic acids, proteins, and small molecules [129–132]. In a similar manner, the target recognition unit in DNAzyme complexes has been designed to be a substrate for DNA repair enzymes. An example of this approach is shown in Fig. 6(a). The sensor is comprised of a chimeric hairpin DNA consisting of the DNAzyme (5′-stem and loop, black) and the blocker RNA (3′-stem, gray). In the presence of RNase H, the blocker RNA is degraded, and the DNAzyme is liberated to catalyze cleavage of MBs labeled with F and Q groups at both ends in the presence of Mg^{2+}, resulting in production of a strong fluorescence signal [43]. The proposal that the DNAzyme itself can be used as a substrate for the target enzyme stimulated the development of a new type of assay for determining the activities of DNA repair enzyme [133,134]. Fig. 6(b) shows an example of this type, in which UDG activity is determined. The catalytic activity of the DNAzyme is recovered upon UDG-mediated removal of the U nucleobase positioned in the DNAzyme, which consequently leads to a significant fluorescence increase through cleavage reactions [44]. Owing to high turnover rates of DNAzymes, these strategies analyze the DNA repair enzyme activities with high sensitivity and speed (Table 1). We expect that this type of sensors could be further reinforced by the combination with other signal amplification strategies (e.g., isothermal nucleic acid amplification), which would enable the label-free and ultrasensitive assay of DNA repair enzymes.

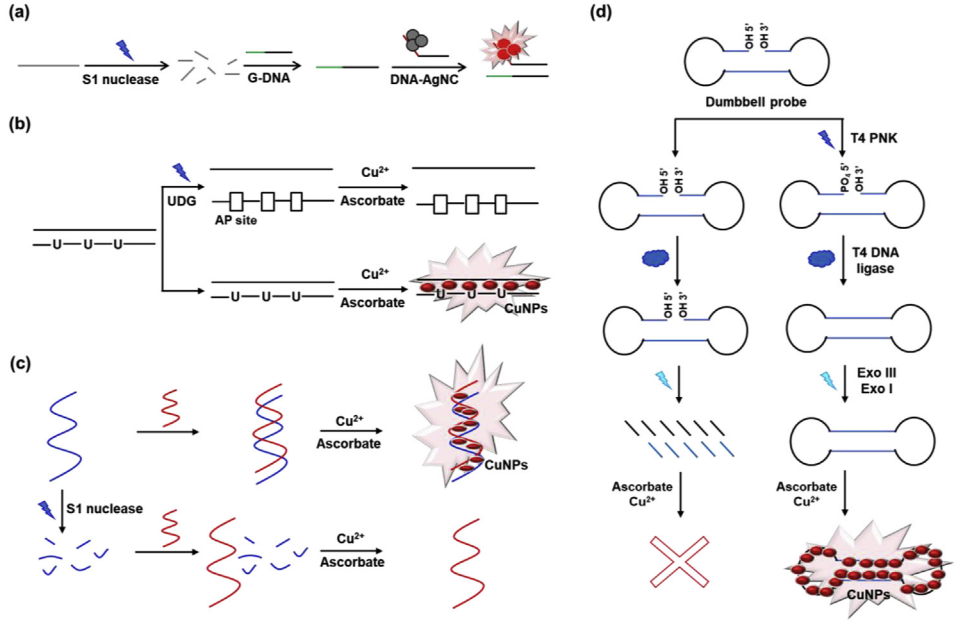

Fig. 5. Fluorescent methods for detection of DNA repair enzymes, which utilize DNA-templated nanomaterials. (a) S1 nuclease activity assay based on the fluorescence enhancement of DNA-AgNCs in proximity to the G-rich sequence [115]. (b) UDG activity assay [113] and (c) S1 nuclease activity assay [112] based on DNA-templated fluorescent CuNPs. (d) T4 PNK activity assay based on dumbbell probe-templated fluorescent CuNPs [116].

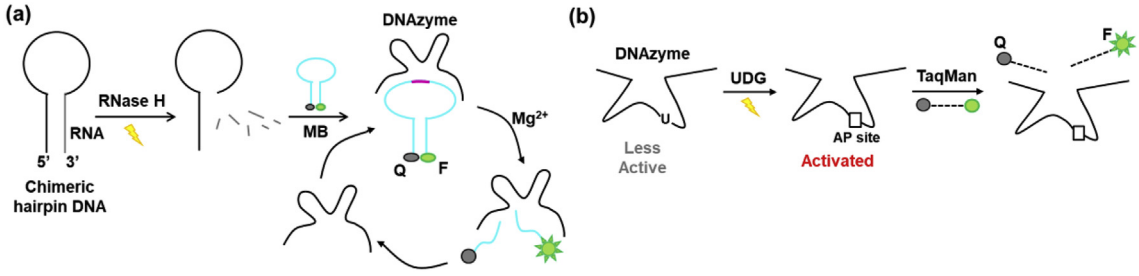

Fig. 6. DNAzyme-based fluorescent assays for (a) RNase H activity [43] and (b) UDG activity [44].

2.7. DNA aptamers

DNA aptamers, nucleic acids possessing strong affinities toward specific target molecules, have emerged as promising alternatives to antibodies [47–50,135]. Especially interesting is a DNA aptamer that binds to and inhibits DNA polymerase activity (DNA polymerase-specific aptamer) because it can be used in sensing systems in which it is coupled to DNA polymerase-mediated reactions [136–140]. Recently, Park et al. found that a DNA polymerase-specific aptamer, engineered to contain a target-specific overhang, regulates DNA polymerase activity by its hybridization with the complementary target DNA [141]. Based on this novel finding, systems have been developed for determining the activities of DNA repair enzymes such as Exo I and UDG (Fig. 7) [142]. As shown in Fig. 7(a), the Exo I blocker hybridizes to the overhang of the Exo I detection probe derived from a DNA polymerase-specific aptamer. The resulting complex (Exo I blocker/Exo I detection probe) binds to and inhibits the DNA polymerase. As a consequence, the inactive DNA polymerase cannot initiate the primer extension reaction and, thus, the TaqMan probe remains in a quenched state. However, when Exo I is present, the Exo I blocker is digested and the destabilized Exo I detection probe cannot inhibit the DNA polymerase. Thus, the active DNA polymerase promotes the primer extension reaction with a consequent fluorescence enhancement from the TaqMan probe. In a similar way, UDG activity was also determined by using a U nucleobase-containing UDG blocker (Fig. 7(b)). Importantly, this approach is highly advantageous in that the target recognition and signal transduction parts

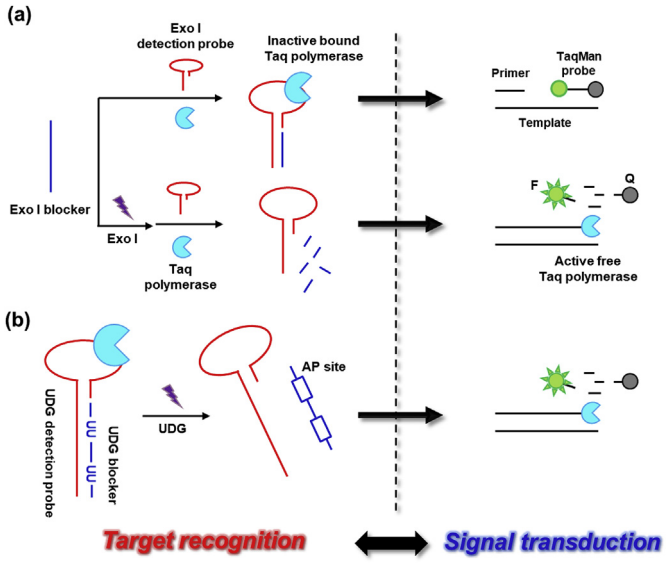

Fig. 7. DNA aptamer-based fluorescent assays for the (a) Exo I activity and (b) UDG activity [142].

are separated. This enables various enzymes to be assayed by simply changing the target-specific overhang while retaining the same signal transduction module [141,142]. It is expected that the discovery of new DNA aptamers that bind to and regulate other enzymes such as strand displacement DNA polymerases or nicking endonucleases would expand the application scope of DNA aptamers and enable the novel and efficient assays that overcome the drawbacks in the previous systems (Table 1).

2.8. Isothermal nucleic acid amplification

Continuing efforts to enhance the sensitivity for the detection of DNA repair enzymes led to the development of a DNA repair enzyme assay that is based on isothermal nucleic acid amplification, a promising substitute for other nucleic acid amplification protocols such as the polymerase chain reaction (PCR). The first system, in which the isothermal nucleic acid amplification method is utilized, was designed to determine UDG activity. The system employs a target-triggered exponential amplification reaction (EXPAR) in conjunction with the RNase H-assisted signal amplification reaction [52,143,144]. Specifically, UDG removes the U nucleobase, giving a product that is subsequently cleaved by Endo IV to generate a free 3′-hydroxyl group. EXPAR then takes place with the action of the DNA polymerase and nicking endonuclease to produce a large amount of ssDNAs that further initiate signal amplification mediated by RNase H. Because of the multiple amplification steps of this sensor, UDG activity can be detected at a level as low as 0.0001 U/mL (Fig. 8(a) and Table 1) [52]. The second example was also designed for the determination of UDG activity. This system employs another isothermal nucleic acid amplification method, termed nicking endonuclease amplification reaction (NEAR) [145–148]. In this system, the U nucleobase is positioned at the cleavage site for the nicking endonuclease (Nt.AlwI) so that the AP site produced in the presence of UDG inhibits the cleavage reaction that is essential for the efficient execution of NEAR (Fig. 8(b) and Fig. S1) [148].

The third sensor of this type is based on RCA that efficiently synthesizes long ss concatemeric tandem repeats at a constant temperature [149–152]. As shown in Fig. 8(c), UDG excises the U nucleobase in the stem of a hairpin probe, which is then cleaved by Endo IV. This process opens the hairpin probe, yielding a protruding strand (orange) that circularizes the padlock probe containing a sequence complementary to the G-quadruplex. As a result, RCA is initiated to produce a number of tandem repeats of the G-quadruplex, leading to highly fluorescent emission from NMM [153]. This method is highly sensitive to UDG activity, but is devaluated by its time-consuming procedures (Table 1). Another interesting system that relies on the RCA, was reported for the determination of RNase H activity [154]. In this method, a specially designed primer containing an RNA sequence (red) in the middle along with a 3′-amino group that blocks the DNA polymerase-mediated extension,

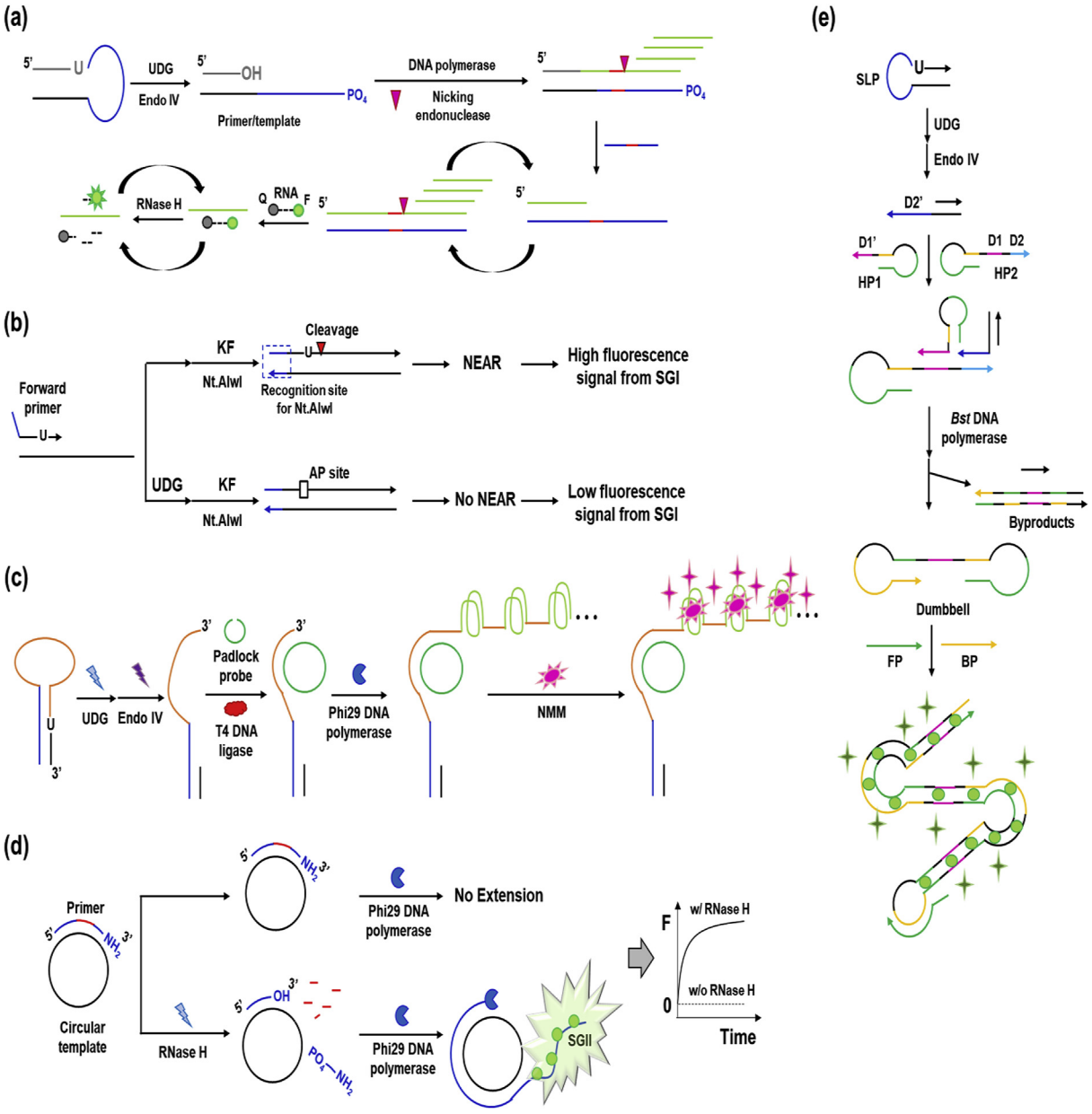

Fig. 8. Fluorescent methods for detection of DNA repair enzymes based on isothermal nucleic acid amplification. UDG activity assays with (a) EXPAR [52], (b) NEAR (for complete scheme, see Fig. S1) [148], and (c) RCA [153]. (d) RNase H activity assay with RCA [154]. (e) UDG activity assay with LAMP (The arrow indicates the 3′-end) [55].

is used as a key detection component. Upon addition of RNase H, the RNA sequence of the primer is degraded and the 3′-amino group is released, being replaced with the 3′-hydroxyl group [154]. This process consequently promotes RCA, which is monitored in real-time with the aid of the ssDNA-specific fluorescent dye, SYBR green II (SGII). By virtue of the real-time monitoring of the isothermal amplification reaction, this strategy can assay RNase H activity in a rapid and sensitive manner (Fig. 8(d) and Table 1) [154].

The fourth sensor of this type is designed by utilizing a stem-loop primer (SLP) that promotes LAMP in response to UDG. As illustrated in Fig. 8(e), the presence of UDG that cleaves the site of U nucleobase with the help of Endo IV, opens SLP to generate the 3′-hydroxyl group, which subsequently binds to D2 region of HP2 and displaces the pre-existing HP1 via *Bst* DNA polymerase-catalyzed extension reaction. As a result, the dumbbell structure that serves as a template for LAMP is formed and upon the addition of primers (FP and BP), LAMP is initiated, leading to the exponential fluorescence enhancement from SGI [55].

In addition to EXPAR, NEAR, RCA, and LAMP, which require enzymes for isothermal nucleic acid amplification, enzyme-free isothermal amplification technologies have been employed. The first class utilizes a catalytic hairpin assembly (CHA) in which ssDNA triggers the self-assembly of two metastable hairpin probes to produce numerous hybridized duplexes through toehold-mediated strand displacement [155–158]. This technique has led to significantly advanced methods for the determination of DNA repair enzyme activities. An example is found in a study for determining T4 PNK activity (Fig. 9(a)) [159]. In this method, the 5′-end of HP is phosphorylated in the presence of T4 PNK, and subsequently digested by λ Exo, producing ssDNA (tDNA) that initiates the CHA reaction. Thus, the resulting tDNA hybridizes to the toehold of HP1, displacing its stem and opening Py-labeled HP1.

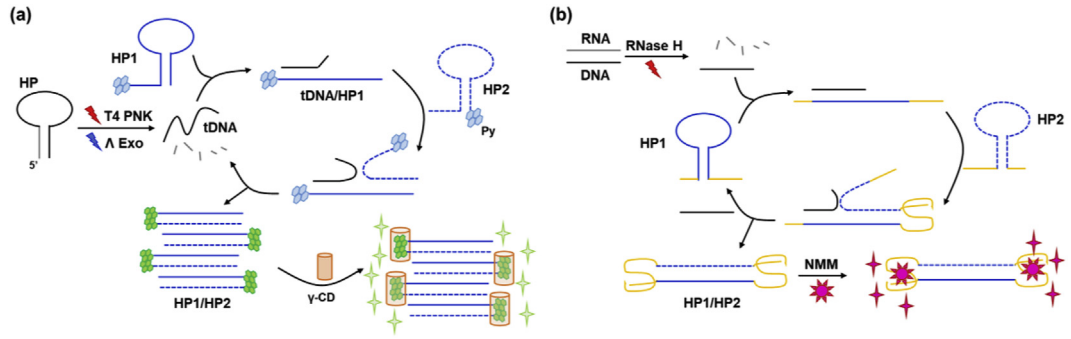

Fig. 9. CHA-based fluorescent assays for DNA repair enzymes. (a) T4 PNK activity assay [159]. (b) Label-free RNase H activity assay [162].

ssDNA of the resulting tDNA/HP1 complex also hybridizes to the toehold of Py-labeled HP2, which forms the duplex, HP1/HP2, while displacing the tDNA. Interestingly, the displaced tDNA promotes multiple cycles of the CHA reaction, leading to the formation of a large number of HP1/HP2s. Consequently, numerous Py excimers are formed to emit enhanced fluorescence at a longer wavelength [160]. To further enhance the fluorescence of excimers, gamma-cyclodextrin (γ-CD), which regulates the distance between Py monomers, is also introduced, consequently enabling the sensitive detection of T4 PNK [161]. In addition, Lee et al. designed a label-free CHA method for determining DNA repair enzyme activity. As shown in Fig. 9(b), this system contains HP1 and HP2 that are ingeniously designed to contain the G-rich sequences at both ends. Thus, promotion of the CHA reaction in the presence of RNase H leads to formation of a large number of G-quadruplexes along with intense fluorescent emission from NMM [162]. This detection principle was also applied to develop a label-free sensitive assay for UDG activity [163].

The second class of this type uses the hybridization chain reaction (HCR) in which ssDNA promotes self-assembly of two metastable hairpin probes to form long chain-like dsDNA products through toehold-mediated strand displacement [164–168]. Xi et al. developed a sensitive UDG activity assay by taking advantage of HCR and the efficient quenching ability of GO [169]. As illustrated in Fig. 10(a), in the absence of UDG, the initiator for HCR (red) is inactive because it is caged by the complement (blue) containing the U nucleobases in HP. Thus, HP1 with the sticky end and HP2 with the F-labeled sticky end are adsorbed onto GO, and thereby significant fluorescence quenching takes place. However, upon addition of UDG, the U nucleobases are removed, which exposes the initiator strand in HP and enables it to invade HP1 and trigger subsequent HCR. As a result, a long chain-like dsDNA, which resists adsorption onto GO, is formed and an intense fluorescence signal is observed. A similar, but label-free approach was also devised to determine the activity of EcoRI. As shown in Fig. 10(b), upon treatment with EcoRI, ssDNA (tDNA) is generated from HP, which triggers the HCR reaction. Thus, long dsDNA products are formed, which is signaled by strong fluorescence from SGI [170]. In addition, another label-free strategy, in which G-rich sequences (green) are incorporated into both ends of each hairpin probe (HP1 and HP2), was devised to detect T4 PNK activity. As illustrated in Fig. 10(c), after successive reactions promoted by T4 PNK and λ Exo, tDNA is released from HP to stimulate the HCR reaction. This process gives rise to the formation of long concatemeric dsDNAs containing many split G-quadruplexes, which leads to significantly enhanced fluorescence from NMM [171]. Interestingly, there was an attempt to integrate two HCR cycles in order to improve the detection sensitivity of UDG activity assay. In the absence of UDG, the initiator strand (I) promotes the upstream HCR reaction (HCR-1) with HP1 and HP2 and produces the long chain-like dsDNAs having a split ssDNA at each nick, which further functions as a trigger (T) for the downstream HCR (HCR-2). Subsequently, T promotes HCR-2 in which HP3, HP4, HP5, and HP6 are sequentially and repetitively hybridized. As a result, the frond-like branched dsDNA nanostructures are generated, thereby giving the effective FRET phenomenon. On the other hand, when UDG excises the U nucleobases of I, it cannot initiate both HCR-1 and HCR-2, and the negligible FRET is displayed. With this novel amplifier, the UDG activity was sensitively assayed with the detect limit of 0.00011 U/mL, which is superior to that of the conventional system that relies on one HCR cycle (Detection limit: 0.0013 U/mL) (Fig. 10(d) and Table 1) [172]. Overall, without the aid of enzymes, the analytical systems resulted in the detection performances comparable or even better to the counterparts relying on enzymes, implying that new enzyme-free isothermal amplification circuits would serve as a compelling alternative to enzyme-based ones (Table 1).

3. Summary and conclusions

DNA repair enzymes play critical roles in genomic integrity, disease outbreak and development of therapeutics. These important features have stimulated the development of numerous analytical methods to detect activities of DNA repair enzymes. In particular, extensive attention has been given to the development of fluorescence-based methods which have intrinsic merits including high sensitivity, simplicity and ease of operation. This review summarized and discussed about these methods, notably focusing on ingenious designs that exploit the unique features of nucleic acids, nucleic acid-templated fluorescent nanomaterials and isothermal nucleic acid amplification (Table 1). Moreover, this review provided insights into strategies that can be employed in devising novel fluorescent sensing systems not only for DNA repair enzymes but also for other biologically important entities.

4. Future perspectives

Despite the fact that advances have been made in the development of novel assays for determining activities of DNA repair enzymes, two unresolved issues remain. The first concerns multiplex analysis of DNA repair enzyme activities, which has not been fully explored owing to technical difficulties [51,173–176]. Specifically, it has been an issue to comprehensively grasp DNA repair enzyme activities and achieve the high-throughput screening of their inhibitors which are of significance for the development of potential therapeutic agents [173,177–179]. Thus far, some preliminary studies aimed at this goal have been carried out. For example, Huang et al. attempted to determine the activities of restriction endonucleases in a multiplex format, utilizing quantum

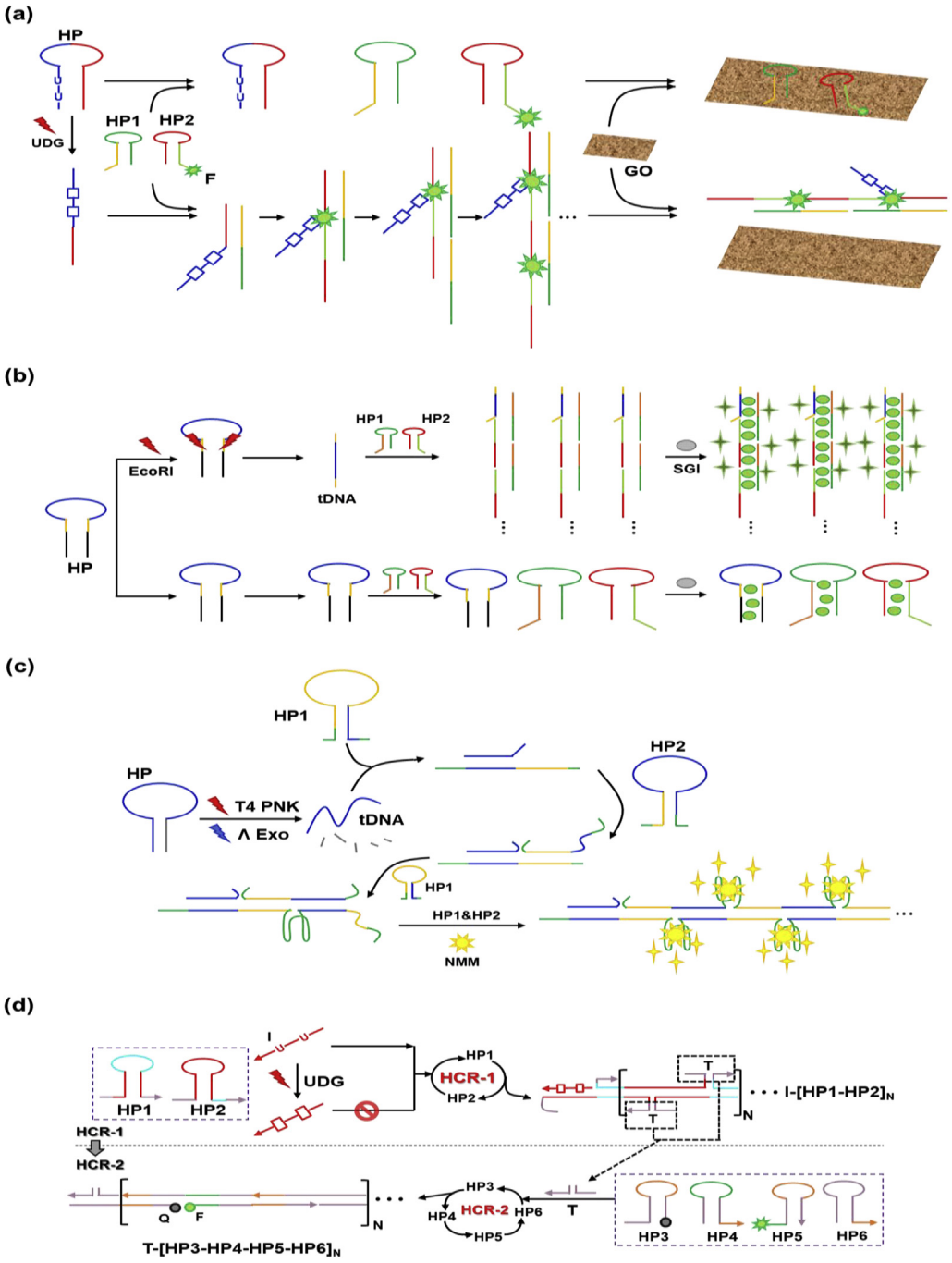

Fig. 10. HCR-based fluorescent assays for DNA repair enzymes. (a) UDG activity assay [169]. (b) Label-free EcoRI activity assay [170]. (c) Label-free T4 PNK activity assay [171]. (d) UDG activity assay with 2-layered HCR [172].

dots with the size-dependent fluorescent properties [174]. In addition, a microarray technology was devised by Flaender et al. for parallel, high-throughput analysis of the BER enzyme activities [173]. However, the number of samples that can be simultaneously analyzed is limited and protocols are too complex to be employed for practical utilization.

The second issue that has not yet been fully explored is *in-vivo* analysis which can accurately reflect real, biological environments in which the enzymes function. It is known that DNA repair pathways are closed associated with the aetiology of diseases such as cancers and AIDS, and relevant enzymes have been regarded as effective targets for the development of potential therapeutics [14,15,180–184]. Until now, a great effort has been focused on direct monitoring of enzyme activities in *in-vivo* environments such as cells or in patient samples. However, various DNA probes designed for *in-vitro* assays fail to work under *in-vivo* conditions, which is attributed to a number of interfering agents present in cells, such as nucleases or DNA binding proteins. Thus, only a few fluorescent probes that work inside cells have been devised to directly measure the expression levels of repair enzymes including ALKBH3, MGMT, APE1, and UDG, indicating that various factors, including cell permeability and unique cellular environments, that are different from *in-vitro* experimental conditions, need to be considered in the construction of new fluorescent probes [26,51,185–188]. One

meaningful observation was made in recent studies of a cellular APE1 assay, which concern the protection effect of silica-coated magnetic nanoparticles against the nonspecific nuclease-induced digestion of immobilized DNA probes [186,189–191]. In addition, Kool et al. succeeded in analyzing ALKBH3 and UDG activities *in-vivo* by designing novel fluorescent probes whose sizes are sufficiently small to permeate into cells to directly respond to target-promoted reactions [187,188,192].

Overall, future efforts should be directed to not only fulfill the unmet needs such as analytical capacity and ease of operation in multiplex analysis, but also devise new DNA probes that can be stably put into real samples and monitor the activities of target enzymes for *in-vivo* analysis, which will undoubtedly prosper the relevant biomedical researches.

Conflict of interest

There is no conflict of interest on this manuscript.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2018.12.055.

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