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Site-selective reactions of imperfectly matched DNA with small chemical molecules: applications in mutation detection

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Abstract

The last decade has witnessed many exciting scientific publications associated with site-selective reactions of small chemical molecules with imperfectly matched DNA. Typical examples are carbodiimide, hydroxylamine, potassium permanganate, osmium tetroxide, chemical tagging probes, biotinylated, chemiluminescent and fluorescent probes, and all of them selectively react with imperfectly matched DNA. More recently, some therapeutic agents including DNA intercalating drugs and groove binders have been found to promote the *in vivo* repair system to recognize and repair the mismatch more effectively. The results have established a novel method for detection of mismatches. Development of new chemical reactions for detection of imperfectly matched DNA and mutations is a rapidly growing field and has attracted significant interest of scientists from both chemical and biological fields and it is the main focus of this review.

Keywords: Imperfectly matched DNA; Mismatched DNA; Unmatched DNA; Abasic DNA; Site-selective reactions of DNA; Detection of mutations

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1. Imperfectly matched DNA

Imperfectly matched DNA contains artificial or natural lesions in which the hydrogen bond donor and acceptor groups on each base within the Watson–Crick double helix are no longer present or not fully exposed for base-pairing. Detection of imperfectly matched DNA is a key to mutation detection and thus a factor for success in prevention, diagnosis, and therapy in our scientific and health care community. From a chemical point of view, imperfectly matched DNA can be classified into four common categories (Table 1): (i) single-base mismatched DNA [1]: DNA contains any pairings between the four bases (A, T, C, and G) which are not the usual Watson–Crick pairing. This category includes eight possible mispairs: A.A, A.C, C.C, C.T, G.G, G.A, T.T, and T.G; (ii) unmatched DNA [2]: one strand of a duplex is longer than the other because of the occurrence of an insertion or deletion in the parent duplex; (iii) modified base containing DNA [3]: when a natural base of a pair has been modified by chemical, physical, or enzymatic means (e.g., methylated bases, thymine glycol, thymine dimer, etc.); (iv) Apurinic and apyrimidinic (AP) DNA [4]: one or more bases have been removed from the DNA by cleavage of glycosidic linkages between nucleotide base and the sugar phosphate backbone. This lesion is commonly referred to as abasic DNA.

The imperfect duplexes are generated in many different ways including spontaneous errors of replication [5], recombination [6], action of chemical or physical mutagens to which the cell or organism is being exposed (e.g., irradiation, deamination of thymine, etc.) [5] and formation of heteroduplexes by mixing wild-type and mutant DNA for diagnostic purposes [7].

2. "Site-selective" factors

Recognition of a small chemical molecule with the imperfect sites located in a DNA macromolecule is regarded as a "site-selective" reaction. Proof for the "site-selective" reaction has been established on three pieces of key information: local conformational changes, physical, and chemical properties of the imperfect duplexes.

Table 1
Terms associated with Watson–Crick and non-Watson–Crick base pairs

Terms used in this review	Changes in a single base pair
Watson–Crick perfect DNA	Complementary base pairs: A.T and C.G only
Mismatched DNA[a]	A.A, A.C, C.C, C.T, G.G, G.A, T.T, and T.G
Unmatched DNA[a]	Extra nucleotide bases on one strand relative to the other
Modified base containing DNA	A normal base is paired with a modified base
Abasic DNA	One base of a pair is removed. The remaining base is thus opposite to the sugar moiety

[a] This category includes the heteroduplex DNA formed by heating and cooling a mixture of wild-type and mutant DNA for mutation detection application.

2.1. Conformational changes

The conformational studies have indicated that the imperfect DNA molecule becomes locally destabilized, hydrogen bonds of base pairs are partially or fully disrupted, the disturbed nucleotide bases are extra-helical and flip out of base stack and the lesion sites become highly susceptible to many enzymatic and chemical reactions [8]. For convenient and effective studies by modern techniques (X-ray crystallographic studies, ^{1}H and ^{13}C NMR and FT-IR), short synthetic oligonucleotide sequences (10–40 bases) containing a desired base are mixed in equal amounts, heated, and cooled for annealing to form sets of heteroduplex DNA. X-ray studies of 12-mer DNA containing a single-base mismatch was reported in the early years of the 1980s [9] and the results indicated that the structural perturbation of the mismatch was localized. Mismatched nucleotide bases became extra-helical and were exposed to the external medium [10]. A similar result (local perturbation) was also obtained from a study of the crystal structure of a 10-bp DNA molecule containing 8-oxo-guanine [11]. Fourier transform infrared (FT-IR) spectroscopy with multivariate statistics [12] was later applied to give direct evidence of the backbone perturbation and the glycosidic torsion caused by this lesion. FT-IR spectral data between the control and the 8-oxo-adenine/guanine lesions in 25-base single DNA strands showed significant differences in absorption at around 1675 and 1233 cm^{-1} (due to antisymmetric stretching vibrations of the PO_2^- group), suggesting that conformational change in the backbone likely occurred immediately adjacent to the base containing the oxo-group.

Parallel studies of the single modified base DNA duplexes using the NMR technique fully supported the common features of local conformational changes as described above. In a typical example, a synthetic 11-bp oligonucleotide [13], which contained one middle thymine residue was oxidized with $KMnO_4$ and the resulting thymine glycol (Tg) containing oligonucleotide was annealed with the complementary strand to form the heteroduplex DNA. Conformation of the mismatch A.Tg was fully characterized using one- and two-dimensional ^{1}H and ^{13}C NMR experiments. The spectral data confirmed that the thymine glycol induced a large conformational change in heteroduplex DNA, where the thymine glycol and its complementary base were not stacked in the DNA but were both extra-helical. This phenomenon was later described in a more dynamic term as ''base flipping'' [14].

The base flipping out of the DNA base stack was further studied in unmatched and abasic DNA structures. The model single-base DNA bulges (unmatched duplexes) [15] have been characterized by NMR and the results indicated that the base either intercalated in or looped out of the duplexes depending on the temperature, the DNA sequence and the neighboring bases [16]. Single purine bases were found to be prone to intercalate into the helix and were less stable than the pyrimidine base bulges [17]. The bulge became the target for selective reactions of DNA repair enzymes, intercalators and other DNA binding drugs [18]. For structural studies of abasic DNA, the duplexes displayed the B-form with only localized perturbation at the lesion sites. The perturbation induced the axial curvature and was larger with unpaired pyrimidines than with the unpaired purines. Base stacking was also influenced by the neighboring bases to the AP sites.

The conformational changes including backbone perturbation, glycosidic bond torsion, hydrogen bond disruption, DNA un-stacking, base flipping, etc. are important factors for the site-selectivity of chemical reactions towards the aberrant bases. Among those factors, base-flipping is perhaps one of the most critical factors as the functional groups of the extrahelical bases are positioned in such a way that it can be more accessible for incoming chemical molecules, solvents and other reagents [19]. Recent studies confirmed that the flipping nucleotide was precisely fitted into an active site within a specific repair enzyme system (mismatch-specific DNA glycosylase) [10].

2.2. Physical properties of imperfect DNA

The DNA duplex, which contains imperfectly matched points, dramatically alters physical properties, such as melting temperature and other thermodynamic parameters (enthalpy and free energy changes) [20]. The common physical property of the imperfectly matched DNA is a lower melting temperature compared to perfectly matched ones, as the lesion sites locally destabilize the DNA helix [21].

For single-base mismatched DNA, independent studies from different research groups have shown the order for mismatch stability is as follows: G.T > G.G ~ T.T ~ A.G > A.A > C.T ~ A.C > C.C [22,23]. The stability of a mismatch is dependent not only on the DNA context but also the specific neighboring matched bases (stacking effect) [24–26]. In addition, the acidity of solutions is also taken into account for stability of single-base mismatches. For example, with an A.C mismatch at low pH (*ca.* 5.0) [26], the N1 nitrogen atom within the adenine molecule becomes protonated and thereby the A.C mismatch dramatically increases its stability due to extra formation of a hydrogen bond with an opposite base.

Comparative studies of the melting temperature between matched and Tg (thymine glycol) containing duplexes showed the melting temperature of unmatched DNA was *ca.* 8 °C lower than the perfectly matched one. Thermodynamic studies based on 18-mer duplexes indicated that there were small differences in the melting temperatures (0–3 °C) between different abasic DNA duplexes [24]. However, the melting temperature of the duplexes containing single AP sites were lower by 9–15 °C relative to the full matched control DNA, and by 4–10 °C relative to the corresponding duplexes in which the AP site was replaced by a mismatched nucleotide base. Thus, the stability of the DNA molecule due to one base pair change is in the order of: matched DNA > single-base mismatched DNA > imperfectly matched DNA associated with AP lesions. Local destabilization as indicated by lower melting temperature implies a preference of chemical reactions to take place at the lesion sites where the hydrogen bonds are partially or fully disrupted [27].

2.3. Chemical properties of imperfect DNA

The physical properties of the imperfect DNA duplexes lead to changes in their chemical properties. Decreased melting temperature results in increased solubility and access of reagents and solvents to the lesion sites. Direct evidence for site-selectivity of chemical reactions at the local imperfect sites within the DNA

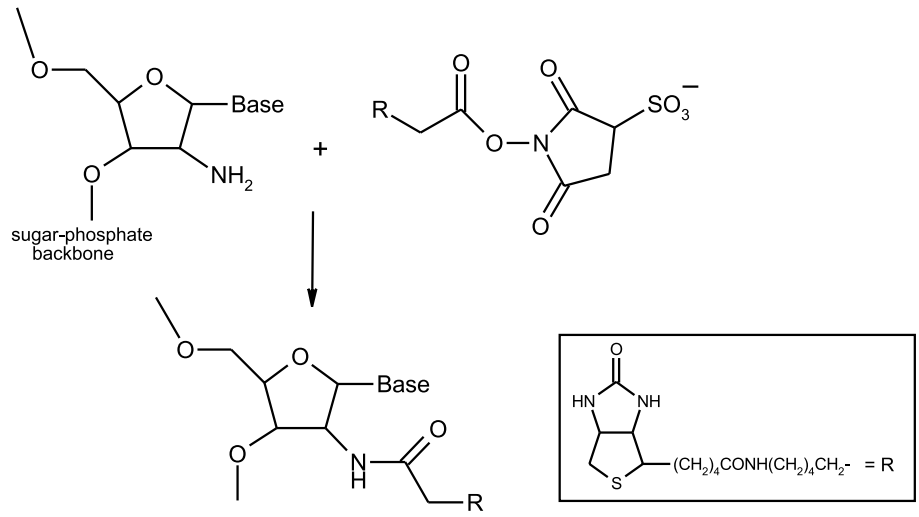

Fig. 1. Detection of mismatched DNA by the tagging method: direct evidence for increased reaction rate at the local imperfect site.

molecule has recently been demonstrated by the chemical tagging method [28]. The method was based on the competitive acylation reaction of a succinimidyl ester molecule (Fig. 1) with sugar-modified nucleotides where the 2′ ribose position was replaced by an amine group. The result clearly showed that the 2′ amino groups at the mismatch site were acylated more rapidly than amine substitutions at base-paired nucleotides. Further experiments were carried out with three types of mismatches (C.T, C.A, and C.C) and all three mismatched duplexes reacted ~30 fold more rapidly than the perfect duplexes at 50 °C. As a consequence, reaction rates are expected to be higher at the mismatched sites than at the rest of the DNA sequence. Based on this study, authors have developed novel acylating agents called biochemical sensors to detect mismatches (the chemical tagging method). The biochemical sensor molecule contains a visual R group that could be biotin, a fluorescent moiety or an enzyme conjugate for various detection platforms. In the case of the bulky biotinyl R-group, the binding site could be detected as a slowly migrating band in a denaturing polyacrylamide gel. This method theoretically should be promising as long as the amino sugar-containing nucleotides are available for DNA amplification.

3. Site-selective reactions of imperfectly matched DNA with small molecules

Detection of imperfectly matched DNA and mutations by chemical means relies on available methods for chemical modification of free nucleotide bases. Free nucleotide bases have been modified in many different chemical pathways but only few of them have been practically employed as a marker or molecular probe in biochemical assays to detect imperfect sites (Table 2).

Table 2
Common chemical modification reactions of nucleotide bases

Type of reaction	Modification site	Reagents
Alkylation and acylation	N1 and N3 of pyrimidine bases and N9 of purine bases	Carbodiimides
	N7 of purine bases	Diethyl pyrocarbonate
Formation of imine	Exocyclic amine (NH_2)	Glyoxal
	Abasic sites (aldehyde)	Hydroxylamine derivatives
Oxidation	Double bonds of pyrimidine bases	OsO_4, $KMnO_4$
Addition and substitution	C4 of pyrimidine bases and double bonds	Hydroxylamine, Methoxylamine
Non-covalent binding	Between base pairs	Intercalators
	Minor or major grooves	Groove binders

3.1. Carbodiimide and its analogues

The carbodiimide (CDI) was first reported to react preferentially with uridylic, thymidylic, and guanylic acid residues within synthetic ribonucleotides and denatured calf thymus DNA in aqueous buffer (pH $\sim$ 8–8.5) [29,30]. The resulting adducts then prevented the uridine-3′ phosphoryl and cytidine-3′ phosphoryl bonds in ribonucleic acid from digestion by pancreatic ribonuclease. This result initiated further applications of CDI to probe the secondary structure of RNA and DNA as well as detection of mismatched G and T bases in DNA heteroduplexes. Detection of mismatches were based on the retardation of the CDI–DNA adduct when the samples were analysed by non-denaturing gel electrophoresis [31]. In the other platform, the bulky carbodiimide group is able to inhibit primer extension at the position of a modified base and the resulting incomplete DNA sequence can be detected on a gel. Such a method was successfully applied to detect unknown mutations in at least three cases in the literature [32–34]. However, the major disadvantages of this method are the requirements of labelling DNA samples and a gel-separation step [35].

The desire to eliminate the time-consuming gel electrophoresis step led to the development of the tagging system such as carbodiimide-containing biotin derivatives as reagents for detecting point mutations [36]. After the regioselective binding of carbodiimide-biotin to a mismatch, a second detector group such as horseradish peroxidase attached to avidin or enzyme-labelled antibiotin antibodies is required to couple with the biotin moiety. When a suitable substrate is added to the reaction mixture, the DNA mismatch can be detected by spectroscopy. Synthesis of the biotin–carbodiimide complex involved the coupling reaction of the commercially available starting material, biotin hydrazine, with bromo-acetic anhydride in dioxane in the presence of $NaHCO_3$. The resulting product, *N*-biotinyl-*N*′-bromoacetylhydrazine, was then reacted with 1-cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide in DMF

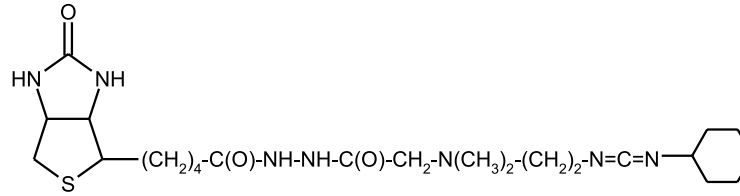

Fig. 2. The biotinyl derivative of CDI for binding to mismatched DNA.

to give the desired compound, which was used for detection of mismatched DNA (Fig. 2).

3.2. Diethyl pyrocarbonate

Base mismatches can take a number of forms, either single or multiple mismatches. The latter is referred as a bubble or bulge where a number of mismatches occurs consecutively and it is fundamentally different from the single base mismatch in terms of its global conformational changes. The sequence exhibited a large gel retardation and the loop-out bases were sensitive to specific chemical reactions [37]. Diethyl pyrocarbonate was reported to selectively react with N7-purine bases at the multiple mismatch sites via a carbethoxylation reaction [38]. The resulting modified sequence was cleaved by piperidine and analysed by denaturing gel electrophoresis. Diethyl pyrocarbonate failed to react with single-base mismatches, but it is a valuable probe for examining the accessibility of particular types of bases in DNA, left-handed Z-DNA, intra-strand paired cruciform structures and conformational changes of DNA induced by intercalators and drug-like molecules [39].

3.3. Potassium permanganate ($KMnO_4$)

Potassium permanganate has been widely used for the oxidation of calf thymus DNA for many years. The reaction was carried out under basic conditions (pH $\sim$10) at 37 °C for several days (or 100 °C for 1 h). Under these conditions, all bases except adenine were completely oxidized to give rise to urea, CO_2, and an alcohol as the final breakdown products [40]. The ease of reaction was found to be in the order: cytosine > thymine > guanine >> adenine [41]. However, under milder conditions (pH = 7–8, at 27 °C for 60 min) the reaction involved the formation of the intermediate cyclic permanganate diester which was immediately decomposed under basic conditions to release the diol, ketone, and the soluble manganese dioxide (MnO_2) as the by-product [42,43]. Selectivity of $KMnO_4$ towards nucleotide bases was later confirmed in a more detailed study where $KMnO_4$ was reacted with the individual nucleotide bases, thymidine, cytidine, guanidine, and adenosine. The rate constants for these reactions were obtained in the order of thymidine > cytidine >> guanidine >> adenosine [44,45]. Due to high reactivity and solubility in water, $KMnO_4$ has been widely adapted to many biochemical assays. The $KMnO_4$ reaction was successfully applied to point mutation detection by independent research groups

Table 3
Site-selective permanganate oxidation reactions used in biochemical assays

Applications	Notes
Detection of single-base mismatches	The chemical cleavage of mismatch method
Detection of proteins (enzymes or hormone receptors) bound to DNA	The footprinting assays. Proteins: polymerase [52], FNR transcriptional regulator [53], Lac promotor [51], glucocorticoid/progesterone receptors [54], etc.
Detection of the interaction between intercalating agents and DNA	Intercalating agents: ethidium bromide, 9-amino acridine, echinomycin, etc.
Detection of thymine dimers	Thymine dimers generated as a result of [2 + 2] photocycloaddition between adjacent thymines
Detection of 8-oxo-guanine	The lesion generated as a result of ionizing radiation or photo-irradiation *in vivo*

[46–48], detection of intercalation-induced changes in DNA structure [49], detection of imperfect DNA containing thymine dimers [50] as well as other foot-printing related applications [51–54]. In these applications (Table 3), high efficient oxidation of pyrimidine residues has been observed at the region where the double stranded DNA samples were locally distorted by interaction with proteins (enzymes or hormones), intercalators (ethidium bromide, 9-aminoacridine), whereas the thymine dimer was inert to this reaction due to an absence of the olefinic double bond. The oxidized DNA was cleaved by the piperidine reaction and the resulting diagnostic fragments were separated and identified by denaturing gel electrophoresis. Site-selective permanganate oxidation reactions of single and double-stranded DNA containing 8-oxo-guanine residues were also reported [55]. The reaction induced damages to the neighboring bases (guanine, thymine, and cytosine) and this application was then developed into a highly sensitive chemical assay for monitoring the common forms (8-oxo-G) of oxidative damage to DNA *in vivo* [55].

3.4. Hydroxylamine and OsO_4/$KMnO_4$

Modification of cytosine with hydroxylamine and its analogues (methoxylamine) has attracted considerable attention due to their mutagenic activity [56] and their applications in mutation detection. Combination of two chemicals, hydroxylamine and OsO_4, were first described by Cotton in 1988 as the simplest chemical mean to detect all mismatches and, thus, all mutations [57]. The method employs two commercially available chemicals, hydroxylamine and osmium tetroxide (or potassium permanganate in later studies), to react with mismatched cytosine and thymine residues, respectively [46,57]. The modified mismatched duplexes are then highly susceptible to cleavage by piperidine. The resulting DNA fragments are simply analysed by denaturing polyacrylamide gel electrophoresis to identify the mismatch sites. This method is currently considered as the chemical method of choice for mutation research because of a number of advantages [46,58]: (i) combination of two modification agents (hydroxylamine and $KMnO_4$) allows detection of all classes of C

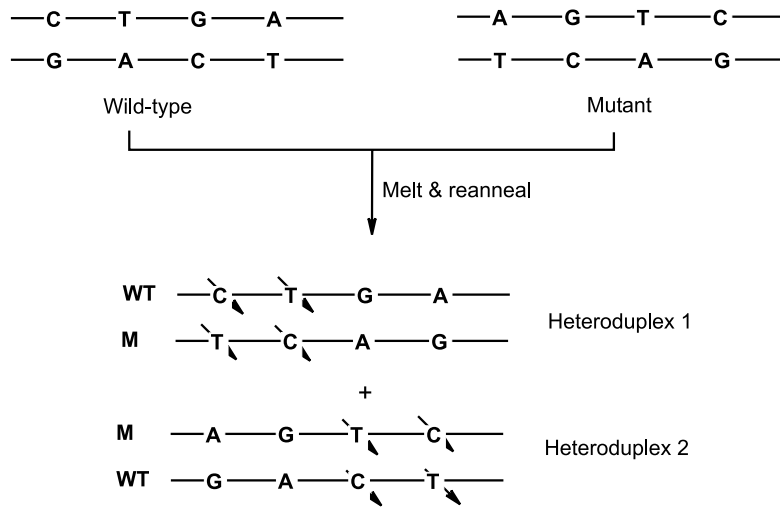

Fig. 3. All mutations have two chances of being detected by use of labelled DNA of both mutant (M) and wild-type (WT) DNA in making the heteroduplexes. Arrows represent cleavage at mismatched T and C bases.

and T mismatches (C.C, C.T, C.A, T.T, T.G, and T.C) and therefore a complete screening of mutation can be achieved (Fig. 3); (ii) if both wild-type and mutant DNA samples are labelled a double chance of detection is possible; (iii) the method is very sensitive to as low as 0.1 μg of DNA sample; (iv) no false positive or negative results have been reported so far; (v) the new solid-phase version of the CCM method has recently made the method simpler [59].

Due to increasing demand in a robust and high throughput system, a simpler detection platform of this method is still required to avoid the laborious gel electrophoresis step and the use of toxic chemicals (hydroxylamine and piperidine).

3.5. Hydroxylamine derivatives

Abasic sites (apurinic and apyrimidinic sites) are common lesions in DNA, which occur spontaneously or can be induced by damaging agents such as ionizing radiation, reactive oxy intermediates, antibiotics, or alkylating agents. From a chemical point of view, the abasic site contains an equilibrium mixture of the cyclic hemiacetals of the deoxy-ribose sugar (>99%) and the open chain aldehyde (<1%) (Fig. 4) [60]. Hydroxylamine and its derivatives can selectively react with the aldehyde functional groups located within abasic sites to form stable oxime moieties. Due to the specific nature of this reaction, abasic sites are highly reactive towards the aminooxy moiety and therefore several hydroxylamine derivatives containing biotinyl or fluorescent R groups have been developed for detection of AP sites [61]. Typical examples are illustrated in Table 4.

The aldehyde reactive probe (*ARP*): ARP was first described as the biotin containing probe (*N′*-aminooxy-methylcarbonylhydrazino-D-biotin) in 1992 [62]. The

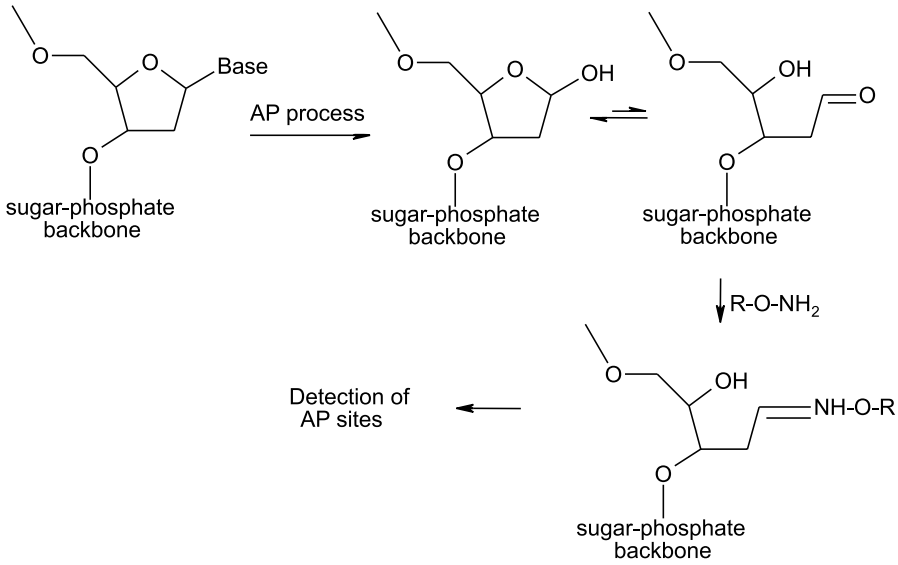

Fig. 4. Reaction of hydroxylamine derivative with the aldehyde group at the AP sites in DNA (R = H or fluorescent moiety).

Table 4
Hydroxylamine derivatives used for detection of abasic DNA

Compounds	Detection method	Sensitivity
The aldehyde reactive probe (ARP)[a]	Biotin-tag/streptanidine–horseradish peroxidase	12 AP sites per 10^6 nucleotides
Fluorescent aldehyde reactive probe (FARP)[b]	Fluorescence excitation (490 nm) and emission (515 nm)	0.6 AP sites per 10^4 nucleotides
Acridinium hydroxylamine (AHA)	Chemiluminescence	0.1 AP sites per 10^6 nucleotides
Dansyl and Lissamine–Rhodamine fluorophores	Fluorescence excitation (323 nm) and emission (550 nm)	1 AP sites per 10^5 nucleotides
O-(Fluoresceinylmethyl) hydroxylamine (OFMHA)	Fluorescence excitation (470 nm) and emission (514 nm)	10 AP sites per 10^5 nucleotides

[a] ARP: *N*′-aminooxy-methylcarbonylhydrazino-D-biotin.
[b] FARP:5-((((2-(carbohydrazino)-methyl)thio)acetyl)-aminofluorescein, aminooxy-acetylhydrazide).

ARP assay can be applied for a wide range of samples including isolated DNA, nuclei and live cells, as the molecule can penetrate the cell membrane. In all cases, the DNA–ARP adduct needs to be isolated before the abasic sites can be quantitated colorimetrically by an ELISA-like assay using an avidin–biotin–horseradish peroxidase and a suitable chemiluminescent substrate [62].

The desire to reduce the use of the cumbersome ELISA-like assay, and to improve the sensitivity for detection of AP sites led to development of a chemiluminescent

compound such as acridinium hydroxylamine (AHA) [63]. AHA directly reacts with the the abasic site and the modified site can be quantitatively determined by a luminometer at very high sensitivity level (0.1 AP site per 10^6 nucleotides). A number of fluorescent probes, which carry the oxy-amino moiety, have also been developed for similar purposes. After being treated with the probes Dansyl and Lissamine–Rhodamine fluorophores [64], *O*-(fluoresceinylmethyl)hydroxylamine [65], fluorescent aldehyde reactive probe [66], the DNA adducts are purified by standard ethanol precipitation methods and subjected to a fluorescent spectrophotometer. The abasic site can be directly quantitated based on luminescent emission level. The sensitivity of each assay is varied and depends on the described experimental conditions in each study.

3.6. Glyoxal

Adenine and guanine selectively react with the carbonyl functionality of glyoxal to give the imine in pyridine at room temperature [67]. The Schiff base formation reaction with glyoxal was firstly applied to modify nucleotides at the B–Z junctions of DNA molecules. The site-selective reaction of glyoxal was later reported successfully to detect non-paired guanine residues within the cruciform structure of DNA but no further application has been published since then [68].

3.7. DNA intercalating/chelating molecules

Development of new therapeutic agents and their non-covalent interaction with DNA have recently become increasingly important for the application of mutation detection. Some DNA intercalating molecules and groove binders have been designed to exploit sequence selectivity and the local destabilization associated with imperfect DNA to detect the lesion more efficiently (Table 5).

A typical example is the novel rhodium intercalator, which has recently been reported to be an applicable probe for single-base mismatch detection [69]. The

Table 5
Common chemicals used for mismatch modification via non-covalent binding mode

Chemicals	Binding mode	Applications
Rhodium complex	Intercalator	Detection of mismatches (C.C, T.C, and C.A)
Nickel (II) complex	Complex formation with guanine base	Detection of mismatch (G.G)
Cobalt(III) hexamine	Major groove binder (GAAA)	Detection of mismatch (G.A)
2-Acylamino-1, 8-naphthyridine	Complex formation with guanine bases	Detection of unmatch (guanine-bulge)
Naphthyridine dimer	Complex formation with guanine bases	Detection of mismatch (G.G)
DAPI	Minor groove binder	Detection of mismatch (T.T)
Immidazole pairs	Minor groove binder	Detection of mismatch (T.G)

rhodium complex, $[Rh(bpy)_2(chrysi)]^{3+}$ (where chrysi = 5,6-chry-senequinone diimine), was synthesized from $[Rh\text{-}(bpy)_2(NH_3)_2]$ $(PF_6)_3$ and 5,6-chry-senequinone. The mismatch detection experiments were successfully carried out with a 2725 bp linearized plasmid heteroduplex containing a C.C mismatch [70], as well as model 17-bp DNA duplexes which contain one single-base mismatch (C.C, T.C, or C.A). The cleavage at the modified mismatched site was promoted by photoactivation (irradiation at 440 nm for 10 min) and the resulting fragments were separated by gel-electrophoresis. The method worked well with C.C and T.C but less satisfactory with C.A mismatches [70].

Development of new metal complexes to probe the DNA structure is a topic of continued pursuit in various research groups as they can provide steric constraints and overall positive charge on the complexes as additional levels to enhance the DNA binding affinity by providing non-covalent electrostatic DNA binding forces. A typical example is the square-planar nickel (II) complex {[2,12-dimethyl-2,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaene]nickle(II)} [71]. The molecule was found to be a highly active oxidant of mismatched guanine residues at the N7 nitrogen atom in the presence of $KHSO_5$ and provided a useful method for structural studies of polynucleotides. The mutation application was successfully carried out on a 15-bp DNA fragment, which contained a G–G mismatch, but the method was limited to the guanine-containing mispairs only. In addition, design of the metal complex such as cobalt (III) hexamine $\{Co(NH_3)_6^{3+}\}$, which is bound to the major groove of the GAAA sequence close to the G.A mismatch is also an innovative sequence-selective approach to improve site-selectivity for detection of mismatches [72]. Conclusive data were obtained from NMR experiments (using the Nuclear Overhauser Enhancement experiments) to determine the structure of the metal complex binding site at guanine base N7 and phosphate oxygen atoms of the tetraloop in the major groove (GAAA) [72]. Similarly, Naito and coworkers [73] have recently developed the intercalating agent, 2-acylamino-1,8-naphthyridine, which accomplishes a complex formation through multiple hydrogen bonds with single guanine bulge (Fig. 5). The

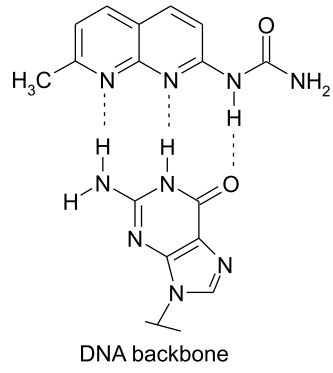

Fig. 5. Complex formation between the 2-acylamino-1,8-naphthyridine molecule (top) and the unmatched guanine base (bottom).

compounds were tested on 52-bp DNA sequences containing G-bulges and the binding sites were confirmed by Dnase I footprinting assay/polyacrylamide gel analysis. This successful experiment led to the development of the dimeric form of the naphtharidine molecule to recognize the G.G mismatches via a hypothetical model of a pseudo G-bulge intermediate and successfully demonstrated on the 9-bp model DNA containing a G.G mismatch [74]. This technology was later developed into a solid-phase approach for detection of ligand-bound G.G mismatch SNP (single nucleotide polymorphism) type [75].

3.8. DNA groove binders

3.8.1. 4′,6-Diamodino-2-phenylindole (DAPI)

The synthetic antibiotic DAPI molecule (Fig. 6) [76] has been used as a DNA-binding drug that interfered with some activities of DNA processing enzymes such as DNA ligase, exonuclease III, and polymerase I. DAPI was also used as a fluorescent dye for staining DNA and chromosomes. The molecule has recently been found to bind selectively to the minor groove of a T.T mismatch-containing site flanked by A.T base pairs. In a model study, it selectively bound in the minor groove of a DNA oligomer $[d(GCGATTCGC)]_2$ containing a central T.T mismatch while no binding was observed inside the G.C tract. The combined results obtained from one- and two-dimensional ^1H NMR spectroscopy, molecular mechanics, and molecular dynamics computations confirmed that a T.T mismatch flanked by A.T base pairs favored the binding of DAPI [77].

3.8.2. Imidazole–imidazole pairs

The T.G mismatched base pair is one of the most common mutations in humans due to spontaneous deamination of 5-methyl cytosine during the replication process. The molecule AR-1-144 (Fig. 7) [78] has been developed to act as a sequence-specific (CTGG) DNA minor groove binder to the wobble conformation of T.G mismatches. High-resolution NMR was used to probe the binding of this drug molecule to various DNA sequences containing T.G mismatches (10-bp DNA duplexes). The results confirmed the hydrogen bond formation between the free amino group of guanine with the imidazole nitrogen atom of the polyamide molecule. In addition, an

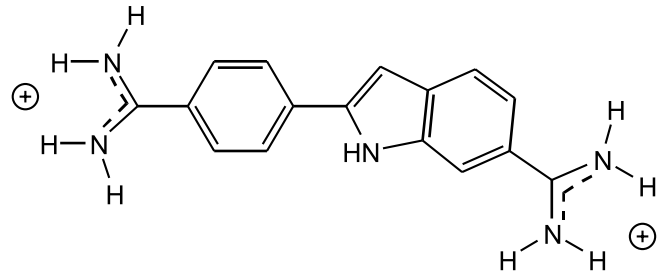

Fig. 6. Chemical structure of the antibiotic, DAPI.

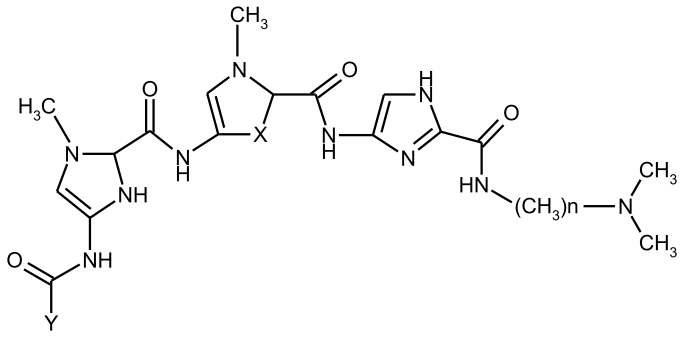

Fig. 7. Im–Im–Im (AR-1-144) X = N, Y = H, $n = 2$, Im–Py–Im X = CH, $n = 4$.

analogue of AR-1-144, the Im–Py–Im molecule (Fig. 7) has recently been described as a good candidate for recognition of specific sequences containing a T.G mismatch.

Although appropriate platforms for detection have not yet been described and other mismatches have not been tested, this pioneer research opens new frontier in search for a new and more effective method for detection of mismatches based on both sequence specificity and site selectivity.

Design and synthesis of the imidazole–imidazole pairs in connection with cyclic enediyne moieties has recently been investigated to improve the sequence specificity of the new class of antitumor antibiotics (enediyne) [79]. Like other members of this drug family [80], the hybrid molecules exert their potent cytotoxic effect by abstracting hydrogen atoms from the DNA sugar backbone via a Bergman cyclisation reaction. The chemotherapeutic application of this class of antibiotic was thoroughly studied in super-coiled plasmid DNA and various cancer cell lines, but their diagnostic applications in mutation detection still remains to be seen.

4. Conclusion

DNA conformational studies, physical, and chemical properties of imperfectly matched DNA provide essential information for understanding the repair processes *in vivo* and site-selective reactions of imperfect matches *in vitro*. Many physical and chemical methodologies are based on the physical and chemical properties of imperfectly matched bases to establish their platforms for detection of damaged DNA. Fundamental chemical work has begun with modification of imperfectly matched bases via covalent or non-covalent bonds. The former requires *bi*-functional molecules where one end reacts specifically with suitable functional groups of the nucleotide bases or the sugar-phosphate backbone of DNA, while the other end is connected to a fluorescent moiety, an enzyme or an antibody for detection purposes. The classical examples of the chemical modification approach are carbodiimide and hydroxylamine with many applications, and some of them are commercially available for detection of mismatched DNA. However, multiple reagents are often required to react with all four types of nucleotide bases and the CCM technology with

two reagents, $KMnO_4$ and hydroxylamine, seems to be an ideal method to cover all eight possible mispairs. Research in the application of intercalators, metal complexes and groove binders are new and very encouraging, as they are highly specific for recognition of mismatched sites. In general, detection of imperfectly matched DNA and mutations is very challenging as the development of a base modifying agent should fulfill a number of important criteria: (i) the reagent should be highly selective towards imperfect sites to avoid side-reactions with matched bases; (ii) the method should be universal for all four bases (or all eight possible mispairs); (iii) the chemical should be a self-detectable compound or *bi*-functional molecule for an attachment of a fluorescent moiety or other detection groups to avoid any subsequent cleavage and gel separation steps; (iv) the chemical should be commercially available, easy to prepare, safe to handle, no requirement of radioactive compounds, and not toxic for further biological assays if required.

Detection of imperfect DNA and mutations is a rapidly growing field and this review describes various chemical approaches based on site-selective reactions with imperfectly matched DNA. Some of them are currently applied in many laboratories and others are still in their infancy. However, they will establish the foundation for the development of new and more effective chemical ways in the most challenging research area of mutation detection. The references used in this review are cited as typical diagnostic applications in mutation detection associated with site-selective reactions and certainly not all-inclusive. The authors sincerely apologize for any valuable publications, which were not included in this review.

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