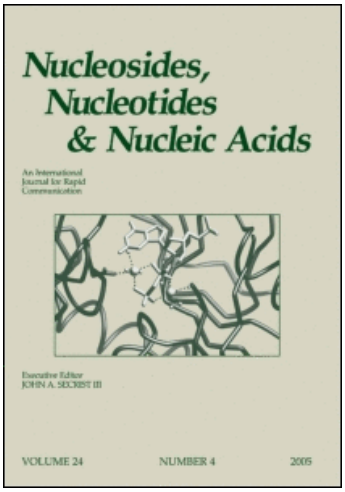

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Permanganate Oxidation Reactions of DNA: Perspective in Biological Studies

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ABSTRACT

$KMnO_4$ has been well known as a powerful chemical probe for numerous applications in biological fields, particularly for those used in conformational studies of DNA. The $KMnO_4$ assay provides essential information for understanding biochemical processes and detecting aberrant DNA, which is associated with many genetic diseases. Elegant examples are sequencing techniques, foot-printing assays for transcriptional studies, an interference method for hormone receptor binding assays as well as DNA conformational studies of Z-DNA, Z-Z junctions, hairpins, curvatures, short nucleotide base repeats, binding of intercalators and groove binders, etc. Recently, $KMnO_4$ has been successfully applied to detect single base changes and mutations in DNA (chemical cleavage of mismatch method, CCM) as well as other types of base damage (8-oxoguanine and thymine dimers). This paper aims to review the usefulness and limitations of the permanganate oxidation reaction used in various biological studies of DNA.

Key Words: Permanganate oxidation reactions; Nucleotide bases; Nucleic acid; DNA; $KMnO_4$ assays.

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1. INTRODUCTION

The use of small chemical molecules for conformational studies of DNA plays a key role in molecular biology.[1] Potassium permanganate ($KMnO_4$) is one of the most effective and useful chemical probes, and it has been applied in numerous applications in biological fields. The preliminary reports[2,3] of permanganate oxidation of DNA appeared in the literature in the early years of the 1960s as a powerful oxidation agent for nucleotide bases and DNA. Since then, numerous $KMnO_4$ applications have been developed, such as sequencing techniques, footprinting assays, DNA interference assays, thymine-dimer assays and the chemical cleavage of mismatch method, etc. This molecule is the chemical of choice for modification of nucleic acids for a number of reasons: (i) $KMnO_4$ is relatively safe to handle. In fact, it was chosen to replace other toxic and notorious oxidants like osmium tetroxide (OsO_4) in various applications (e.g., CCM).[4] (ii) The reagent is very soluble in aqueous solvents which are compatible with many biological buffers (solubility in water is 40–50 g/L at 5–10°C).[5] (iii) The molecule is relatively inert to the intact DNA molecule under mild reaction conditions, but highly reactive towards unstacked bases (denatured DNA). Therefore, it is considered as a probe for single stranded DNA.[6] (iv) It selectively reacts with the double bond of pyrimidine bases (thymine and cytosine) without disturbing other sugar and phosphate moieties within the DNA duplex. (v) The permanganate oxidation of DNA is sequence-selective. As an example, $KMnO_4$ selectively reacts with thymine at the junction between the A-track and the flanking DNA[7] while some non-selective probes (e.g., dimethyl sulfate, DMS) react uniformly with the susceptible bases irrespective of the position of the bases in the sequence.[8] (vi) The permanganate oxidation reaction can be carried out under in vivo conditions, where $KMnO_4$ selectively reacts with the DNA molecule inside the cell without interference of other metabolites.[9] This application puts the molecule in an outstanding position as a suitable method for direct probing of the chromosome in the cell (only OsO_4 and $KMnO_4$ are currently available for in vivo studies). In addition, the $KMnO_4$ approach is cost-effective and simple to manipulate as compared to other expensive and complicated enzymatic or immunological assays. It, therefore, proves itself to be an extremely suitable chemical probe for many biochemical assays. This paper has focussed on the $KMnO_4$ reactions of nucleotide bases and DNA, which form the basis for the current assays in the biological field. Some critical questions and their limitations associated with this reaction will be included.

2. THE PERMANGANATE OXIDATION REACTIONS OF NUCLEOTIDE BASES AND DNA

Most investigations have been exclusively focussed to the mechanism of the permanganate oxidation reactions of free nucleotide bases,[10] which showed a close similarity to the permanganate oxidation of other olefins in non-aqueous solvent.[11] In general, $KMnO_4$ selectively reacts with the double bond of pyrimidine bases under alkaline conditions to afford the corresponding diols as single products (Fig. 1).[12] However, under neutral or slightly basic conditions (phosphate buffer, pH = 6.8–7.1,

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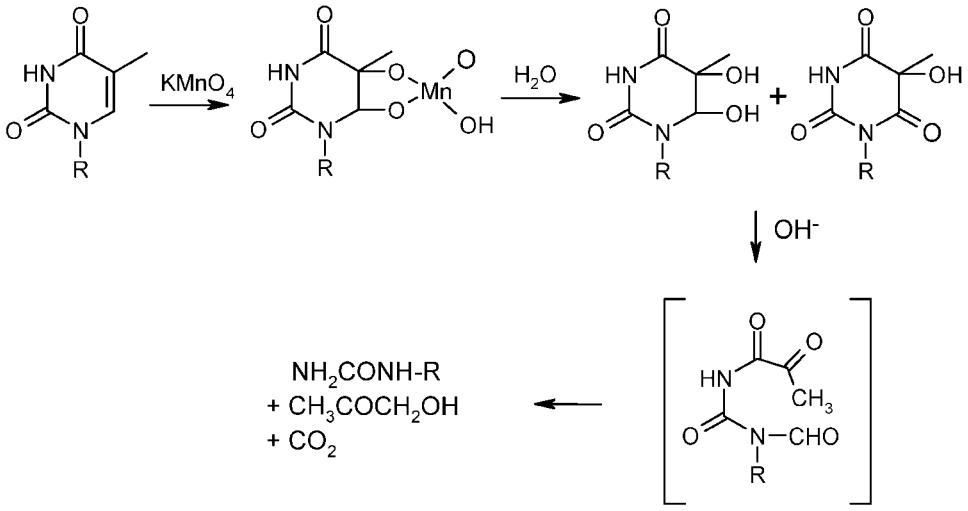

Figure 1. Permanganate oxidation of thymine (R = H or 2′-deoxyribose moiety).

at 15–17°C), a (1:1) mixture of a diol and an alpha-hydroxylketone is obtained.[13] The reaction involves a formation of an intermediate cyclic ester, which is unstable and rapidly decomposes to give the corresponding products as well as the soluble by-product MnO_2.[13,14] When the reaction was incubated under harsher conditions (3 g $KMnO_4$/1 g pyrimidine base, 37°C, pH = 9.0 for 19 h to several days incubation) the reaction proceeded until completion and it usually ended up with fully oxidized products such as CO_2, acetol, and urea (Fig. 1).[3,15,16]

Rates of the oxidation reactions and the oxidation number of the manganese species have been thoroughly investigated in the reactions of uracil and thymine via spectroscopic methods. The oxidation spectra displayed a disappearance of the starting material $KMnO_4$ (absorption at 525 nm) and an appearance of the soluble colloidal MnO_2 (absorption at 420 nm) via the formation of the cyclic ester intermediate (Mn^{+5}). The formation of the five-member cyclic activated complex is the rate-limiting step and it contributes to a strong absorption band at either 280 nm[17] or around 420 nm.[10] These interpretations have been controversial until now.

The reactions proceeded slightly faster with thymine compared to uracil due to the effect of the methyl group substitution at the double bond site.[11] Further investigation of $KMnO_4$ consumption in solutions of nucleosides indicated that the reaction preference was in the order of thymidine > uracil > cytidine ≫ guanosine and no reaction with adenosine.[18,19] These results were confirmed by an oxidation study of free nucleotide bases as well as the oxidation-hydrolysis of the intact DNA molecule. As a typical example, the strong oxidation of calf thymus DNA produced urea and CO_2 as well as unreacted adenine.[15] Recently, permanganate oxidation of purified plasmid DNA was reinvestigated[20] under very mild conditions (0.015–1.5 nM $KMnO_4$ in 0.3 M ammonium acetate, pH 8.6 at 4°C for 5 min). The oxidized DNA was hydrolyzed by formic acid and the resulting products were analyzed by gas chromatography-mass spectroscopy (GC-MS).

Surprisingly, a substantial amount of oxidized forms of all four bases were obtained: both thymine and cytosine were converted into the corresponding diols and dehydration products (hydroxy ketone compounds) as usual. The adenine and guanine molecules were modified into 8-hydroxyadenine and 8-hydroxyguanine respectively as well as small quantities of their ring opening products (Fapy-adenine and Fapy-guanine) (Fig. 2). The mechanism of $KMnO_4$-mediated 8-hydroxyadenine and 8-hydroxyguanine formation is unknown and these results are contrary to the most recent report.[21]

Since the genotoxic activity of $KMnO_4$ and the mutagenicity of the resulting oxidized DNA are important in many biological process,[22] attempts to identify a cis-thymine glycol moiety in the oxidized DNA was carried out.[23] A small fragment of DNA (11 bp) containing a thymine-diol residue was also synthesized and its structure was fully characterized by ^1H & ^{13}C-NMR (nuclear magnetic resonance) experiments.[24] Unlike DNA substrates, the single stranded DNA is highly reactive towards $KMnO_4$ due to the fully exposed nucleotide bases. Chemicals or reagents that unwind the DNA duplexes induce reactivity of $KMnO_4$ at the local disturbed sites. The reactive site has a lower melting temperature and nucleotide bases flip out of the duplex axial, which is referred to as a "bubble".[25] This phenomenon is common for some biological processes such as DNA transcription, replication and repair processes. At the transcriptional bubble, the rate of thymine oxidation close to the transcriptional starting point was found to be higher (up to 3–4 fold) compared to the rate further downstream. The variation is due to the shielding effect of Mg^{2+} ions on the negatively charged groups of DNA and thus lowering the repulsive forces with the MnO_4^- anions.[26] Recently, kinetic studies on the permanganate oxidation reaction with DNA containing damaged sites (8-oxoguanine) was examined.[27] The results indicate that the reaction mechanism is complicated due to the formation of a highly reactive intermediate, which triggers a chain reaction with neighboring bases close to the aberrant sites.

Although the mechanism of the permanganate oxidation reactions of DNA has drawn significant attention from various other research groups (Table 1) over many years, some fundamental issues have not been fully investigated:

(i) The nature of the permanganate oxidation intermediate is still debated whether it is the soluble Mn (IV) species or the long-sought elusive cyclic hypopermanganate (V) diester.[17]

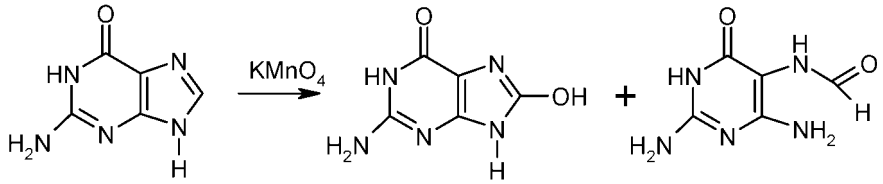

Figure 2. Permanganate oxidation of the guanine moiety in plasmid DNA.

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Table 1. Investigation of the mechanism of the permanganate oxidation reactions of nucleobases and DNA.

Studies of the $KMnO_4$ oxidation reactions	References[a]
Oxidation of DNA (calf thymus, plasmid, etc.)	Jones et al. (1964),[15] Akman et al. (1990)[20]
Oxidation rate & mechanism for substituted olefin and nucleobases	Benn et al. (1960),[2] Chatamra et al. (1963),[3] Freeman et al. (1975),[12] Ogino et al. (1990),[17] Hayatsu et al. (1969, 1991)[10,18]
Purification and characterization of the oxidized duplex DNA	Frenkel et al. (1981),[23] Kao et al. (1993)[24]
Genotoxic activity of $KMnO_4$ in acidic solutions	Meo et. al. (1991)[22]
Kinetic study of permanganate oxidation of 8-oxoguanine in DNA (12 bp)	Koizume et al. (1998)[27]
Quantitative $KMnO_4$ footprinting study, Kinetics of the reaction on DNA	Lozinski et al. (2001)[26]

[a]Studies in olefins other than nucleotide bases and DNA are not included.

(ii) It has been widely accepted that $KMnO_4$ is the chemical probe of pyrimidine bases only. However, its reactivity towards the purine bases (adenosine and guanine) was also reported. The mechanism of purine oxidation has not been fully elucidated and the resulting oxidized products need to be fully characterized.

(iii) The reaction mechanism of DNA oxidation has not been fully elucidated, as it is complicated by multiple reactive sites as well as the heterogeneity of DNA.

(iv) The effect of the other factors (buffer, bases, pH, temperature, DNA and $KMnO_4$ concentrations, etc.) in the reaction conditions have not been fully investigated. As a consequence, the reaction condition requires exhaustive optimization studies prior to each individual assay.

(v) Basic kinetic and thermodynamic data of the oxidized DNA are currently not available.

3. BIOLOGICAL APPLICATIONS

The fundamental concept of the $KMnO_4$ assay has been established based on the site-selectivity of $KMnO_4$ reactions (local denatured or perturbed regions) over a long stretch of a DNA macromolecule.[28] The fully complementary section of the DNA molecule is relatively resistant to the permanganate oxidation under mild reaction conditions while the reactive site is highly prone to $KMnO_4$ reactions. The modified site is then cleaved by various reagents (NaOH, piperidine or

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various diamine derivatives).[29] The resulting DNA fragments are separated by denaturing gel electrophoresis or chromatography. In another detection platform (primer extension analysis), a modified base is able to inhibit primer extension and the resulting non-complete DNA sequence can be detected on standard sequencing gels.[6] Sites of modified residues (thymine) can be quantitatively identified if the primer sequences were labeled by fluorescent groups or radioactive materials.

Several such reactive sites are of interest in biological studies. They include transcription and replication regions, hormone-receptor binding sites, intercalator-DNA interaction sites, drug-bound regions, damaged sites or mismatched base pairs, etc. Proof for the site-selective reactions of $KMnO_4$ has been built on the evidence of conformational changes as well as the physical and chemical properties at these reactive sites.[30,31] In general, nucleotide bases in these sites are extra-helical and flip out of the base stack, hydrogen bonding is disrupted, the glycosidic bond angle is changed and the local disturbed bases become susceptible to chemical and enzymatic reactions.

Based on the principle of the site-selective reaction, the $KMnO_4$ assay was designed to probe the conformational changes within the reactive sites in different applications. In terms of structural studies of DNA, the $KMnO_4$ applications can be grouped into six major categories (Table 2): determination of base composition & DNA sequence, detection of DNA conformational changes induced by protein and small organic molecules, detection of specific structures of DNA, detection of aberrant DNA, detection of mismatches and spectroscopic assays.

Table 2. Biological applications of $KMnO_4$.

Application categories	Biological significance
Determination of base composition and DNA sequence	Determination of adenine composition, thymine-specific DNA sequencing technique, morphological study of chromosomes, contour chromosome delineation technique
Detection of DNA conformational changes induced by protein and small organic molecules.	Foot printing assay: enzyme bound to DNA, interference assay for glucocorticoid receptor and progesterone receptor, detection of intercalator binding site, drug bound DNA
Detection of the specific structures of DNA	Detection of Z-DNA, B-Z and Z-Z junctions, hairpin structures, DNA curvatures, short A tracks, parallel stranded DNA
Detection of aberrant DNA	Detection of thymine dimers, 8-oxoguanine sites
Detection of mismatched DNA & mutations	Chemical Cleavage of Mismatch method (CCM)
Spectroscopic assays	Quantitation of drug-like molecules (ie., naltrexone, phenytoin, carbamazepine) and other bioactive molecules (nucleotide bases)

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3.1. Determination of Base Composition and Sequence of DNA

Determination of the composition of adenine residues within the DNA molecule was one of the first applications of $KMnO_4$.[32] It was established on the principle of the permanganate oxidation-hydrolysis of DNA. Treatment of DNA with $KMnO_4$ at 37°C (pH = 9, for 19 h) oxidized all the bases except adenine. The resulting oxidized DNA was then hydrolyzed under basic conditions (by boiling with KOH or NaOH for 1 h) and fractionated by chromatography. The unreacted adenine was quantitatively determined by colorimetric analysis. Using this technique the percentage of adenine composition was successfully determined in various DNA samples (calf thymus and other bacterial DNA). As the molecule was highly reactive towards thymine under mild reaction conditions, Rubin and Schmid[33] developed the thymine-specific sequencing technique. The DNA sample was briefly treated with $KMnO_4$ (40 mM at 22°C for 15 min) and the reaction was terminated with allyl alcohol. The thymine-modified DNA was cleaved by piperidine and the resulting fragments were separated by denaturing acrylamide gels for sequencing analysis. Due to its simplicity and high sensitivity, this method is frequently used in parallel with other chemicals for sequencing purposes (hydroxylamine for cytosine, dimethyl sulfate for guanine and sodium formate for guanine and adenine).[34]

The strong oxidation property of $KMnO_4$ was also employed in morphological studies of chromosomes as a fixative reagent for nuclear chromatin, the nucleolus and the matrix of mitochondria,[35] as well as the cytological procedure for improving the contour-delineated and non-distorted human chromosome groups by removal of excess DNA.[36]

3.2. Determination of DNA Conformational Changes Induced by Protein and Small Organic Molecules

The initial step for DNA replication and transcription is the binding of an initiator to the promoter (origin) site. The resulting DNA complex forms a "bubble" or opening site, which is remarkably common in both eukaryotic and prokaryotic cells.[37] The opening site is also considered a natural occurrence of negative supercoils (Z-DNA formation) due to an unwinding action induced by enzyme or binding proteins. Hydrogen bond disruption between base-pairs leads to the exposure of nucleotide bases (thymine), which become reactive towards $KMnO_4$. The modified thymine is cleaved by piperidine, followed by fragment analysis or primer extension ($KMnO_4$ footprinting assay, Fig. 3). This assay has been carried out in various biological studies at multiple DNA origins during the transcription or replication processes (Table 3). They include *E. coli* (oriC),[38] the Epstein-Barr virus origin (oriP),[39] plasmid DNA (pjF1 and pBR322),[40] plasmid RK2,[41] yeast replication sequence (autonomously replicating sequence 1, ARS1),[42] transcription promoter of salmonella typhimurium,[43] etc.

The assay can be carried out under both in vitro and in vivo conditions. In in vitro experiments, the purified DNA fragments (100–500 bp) were pre-incubated with the initiator in suitable buffers (Tris-HCl, pH 7.9). The binding solution was

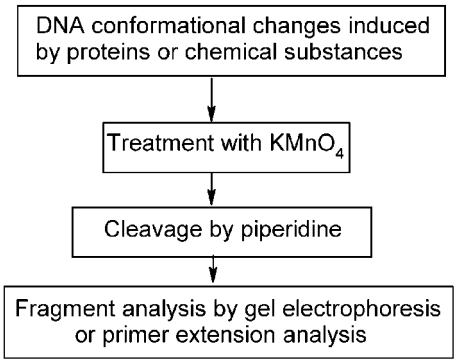

Figure 3. Principle of the $KMnO_4$ footprinting assay.

then treated with $KMnO_4$ (3–10 mM) at 30–45°C for few minutes. The reaction was then stopped by ß-mercaptoethanol (1 M) and DNA was obtained by ethanol precipitation. The resulting DNA was cleaved by piperidine before being subjected to the primer extension analysis or loaded onto a sequencing polyacrylamide gel. In the latter case, the fragments were analyzed in conjunction with Maxam-Gilbert sequencing bands as position markers. Therefore, the binding site could be located and the size of the opening sites could be quantitatively measured.

Table 3. Applications of the $KMnO_4$ footprinting assay in various DNA sytems.

DNA	Origin/initiation protein	Biological study
Epstein-Barr virus	OriP/EBNA1 protein	Evidence for untwisting or bending of the secondary structure of DNA during replication.[39]
Escherichia coli	OriC/DnaA protein	Conformational changes at the origin during replication.[38]
Plasmid RK2	OriV/TrfA and DnaA proteins	Role of TrfA and DnaA proteins for opening of DNA during replication.[41]
Plasmid (pjF1 & pBR322)	Plasmid DNA/RecBCD protein	Development of a model for RecBCD initiation and unwinding complexes.[40]
Salmonella typhimurium	FliC (flagellin gene)/Eσ28 (enzyme RNA Polymerase carrying the σ factor)	Characterisation of the transcription initiation.[43]
Yeast (Saccharomyces cerevisiae)	Origin ARS/SV40T antigen or HSSA binding protein	Mechanism of the replication process.[42]
Plasmid pGS422	Mel/R-lacZ/FNR protein	A role of iron in transcriptional activation.[44]
DNA extracts from cell lines (Hela, XP-A, XP-G)	Lesion site/XPG protein	A role of XPG protein during the repair process.[45]

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The $KMnO_4$ footprinting assay has been extensively carried out in in vivo conditions. The cultures of bacteria were directly incubated with $KMnO_4$ (3–10 mM) at 37°C for 1–5 min. DNA was then extracted and treated with piperidine or amplified by PCR (polymerase chain reaction). The DNA cleavage fragments or incomplete extension products were analyzed by sequencing gels as usual. Comparative studies confirmed the sensitivity of the assay in both in vitro and in vivo conditions, as identical desired fragments were obtained on the same DNA templates. Using this protocol, the role of iron and the binding sites (thymines at the positions −11, −9 and −8 relative to the transcription starting point) of the FNR proteins on the FF-melR promoter could be identified in an anaerobic culture of *E. coli*.[44]

Formation of the unwound DNA bubble around a lesion provides a "recognizable" site for incision by nucleases during the nucleotide excision repair (NER) pathway in eukaryotes. The evidence for local opening of the DNA around the damage site prior to strand scission was carried out by the $KMnO_4$ footprinting study. In a model study,[45] sites sensitive to $KMnO_4$ modification precisely indicated the opening of DNA spanning a ~25 bp region around the cisplatin-DNA lesion, which was the subject for cleavage by the endonuclease enzyme.

Conformational changes of DNA can also be induced by chemical substances such as intercalators or drug-like molecules (groove binders).[46] Detection of intercalation-induced changes in DNA structure as well as identification of binding sites can be rapidly identified by $KMnO_4$. Binding of the intercalator (e.g., ethidium bromide) induces changes in the DNA conformation, causing the DNA to become hyperactive towards permanganate oxidation.[47] However, reactivity was found to be confined to specific intercalators and sequences. For example, $KMnO_4$ displayed thymine hyperactivity with diaminoacridine but not with 9-aminoacridine. The reaction was also confined to specific binding sites of echinomycin,[48] which change as a function of drug/DNA ratio. Comparative studies were also carried out with other currently available chemical probes (diethyl pyrocarbonate, DEPC) to detect echinomycin-induced conformational changes in DNA. Surprisingly, DEPC was less sensitive towards the drug-induced DNA conformational changes compared to permanganate anions. This neutral DEPC molecule seems to require larger scale helix opening while $KMnO_4$ involves transient unstacking events. With this advantage, $KMnO_4$ was proposed to be a sensitive probe for sequence and drug-dependent binding assays.[48]

A desire to investigate a close interaction between binding proteins and nucleotide residues led to the development of the interference assay.[49] Unlike the footprinting assay, which targets the conformational change of the binding site, the interference assay indirectly confirms the intimate contact between the protein and the binding site. Thymines within a single stranded DNA sequence are modified by $KMnO_4$. After reannealing, the modifications interfere with binding of enzymes or hormone receptors only at the positions where they form an intimate contact with thymines (Fig. 4). The resulting DNA derived from the binding assay was recovered and incubated with piperidine (1 M, 30 min at 90°C) for fragment analysis. The interference is indicated as a missing band from the protein-DNA complex on a sequencing gel. This highly sensitive technique yields reliable and specific information on the intimate contact between the protein and the binding site. In this way,

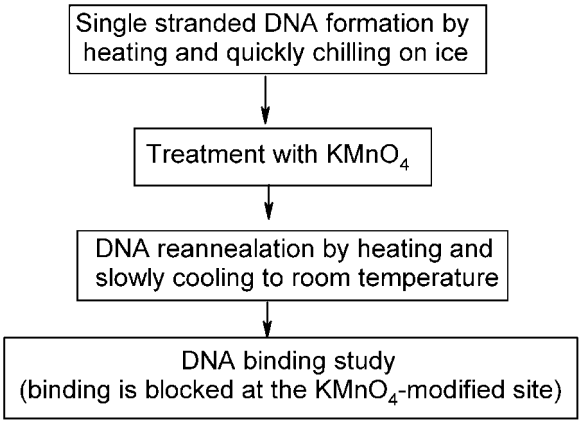

Figure 4. The $KMnO_4$ interference assay.

contact between steroid hormone receptors and thymines (at the positions 10, 12 in the sense strand and position 6 in the anti-sense strand of the DNA fragment containing the hormone responsive element) were identified.[49] The specificity of the assay was also confirmed by analyzing the effect of substituting the particular thymine by different bases. Substitution of thymine 10 by a deoxyuridine reduced the affinity for progesterone and glucocorticoid receptors by 2- and 10-fold respectively. These independent experiments confirmed the accuracy of the $KMnO_4$ reference assay and also demonstrated that only the 5′-methyl group of thymine at this position is relevant for receptor binding.

However, in the other binding study of the GCN4 protein-DNA complex (GCN4 is a member of transcription factors in eukaryotic cells), the $KMnO_4$ interference assay was reported to be less sensitive[50] as only one out of two core thymines at the binding sites was successfully detected.

3.3. Determination of the Specific Structure of DNA

$KMnO_4$ is known as a sequence-selective chemical probe and widely used in studying DNA variations other than the normal Watson-Crick double stranded helix DNA (B-form), such as Z-DNA, curvatures, hairpins and the parallel stranded helix.

Z-DNA. DNA can exist in a left-handed helical conformation with characteristic zigzag tracking of the sugar-phosphate backbone (Z-conformation DNA). The natural occurrence of the Z-structure is believed to be important for living cells as it could be involved in gene regulation events.[51] As a consequence, a quantitative $KMnO_4$ assay has been developed for the analysis of potential functions of left-handed DNA in vitro and in vivo. One typical model study of Z-Z and B-Z junctions was constructed in a plasmid DNA containing two inserts (13 bp, dC-dG repeats). The region between the two inserts contains a BamHI susceptible site and represents the Z-Z junction while the regions outside the inserts contains the EcoRI susceptible

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sites and represent the B-Z junctions.[52] When treated with $KMnO_4$, the restriction site located at these junctions was substantially modified and became immune to the corresponding restriction enzymes. Similar protocols were also applied for the in vivo assay using growing *E. coli* cultures.[52] The cells containing plasmid (Z-Z junction) DNA as described above were grown to late log-phase and treated with $KMnO_4$ (5 μM for 4 min at 34°C). The resulting permanganate adduct DNAs were isolated from cells and mapped with restriction enzyme analysis (EcoRI). The fragments were analyzed by agarose gels and quantified by densitometric scanning with a densitometer. Comparative studies with other probes like osmium tetroxide demonstrated the superior properties of $KMnO_4$. The redox potential of $KMnO_4$ is strongly dependent on the pH of the bacterial medium. Selectivity and sensitivity of the assay can simply be optimized by adjusting the pH value of the culture medium.[52] In addition, the reaction time of $KMnO_4$ is faster than other known oxidant probes even with milli-molar concentrations and there is no need for special precautions during handling. Therefore, the $KMnO_4$ probe is the chemical of choice for studying distorted conformations of DNA within live host cells. However, the mechanism of the uptake of this chemical into the cells has not been identified.

DNA Curvature. DNA curvature with a repeated A-track was found in a wide variety of biological systems of functional importance, such as origin of replication, promoters, etc. The intrinsic feature of the A-track is considered as the "junction model" to describe the physical nature of the bending locus.[53] In a model study, a short A-tract embedded in a fragment of plasmid DNA (*ca.* 200–240 bp) displayed a distinctive conformation, and thymine residues on the complementary section are protected from $KMnO_4$ oxidation. The result suggested a stacking effect within the A-tract regions compared to other random sequences. However, the 3′ side of the poly(dT) strand where the A-track structure clashes with the flanking B-DNA helix showed some reactivity.[7] Further DNA structural studies confirmed the presence of CA/TG steps in this junction section.[54] The model of the junction was reconstructed in dodecamers, [$d(GA_5C_6)$ and $d(C_6A_5G)$] and treated with $KMnO_4$ at low temperatures (4°C). The results indicated that the oligomer $d(GA_5C_6)$ did not react with $KMnO_4$ while the oligomer $d(C_6A_5G)$ containing the T at the CA/TG junction showed remarkable reactivity with $KMnO_4$. This result indicates a large destacking at CA/TG steps in the oligomer $d(C_6A_5G)$, making the T residue at the junction more accessible to the probe. The local destacking nature at the junction containing the CA/TG step was also confirmed by lower melting temperatures (by ca. 4.5°C) as well as smaller changes in enthalpy and free energy (indicating lower thermal stability).[54]

Hairpin Structures of DNA. The trinucleotide (triplet) repeat $\{d(CGG)_n\}$ has been known to be present in mental retardation disorders and other related diseases (Fragile X syndrome, Kennedy disease, Huntington disease, myotonic dystrophy, etc.).[55] Typically, an individual afflicted with one of those diseases contains greater than 50 repeats within a specific gene, compared to a normal individual containing only 5 to 30 repeats. The repeat units of the trinucleotide potentially form hairpin structures, which can block transcription or delay the replication process. The structure of the hairpin is considered as a single stranded sequence or a loop-forming

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structure. Due to the nature of a large scale denatured sequence, the hairpin structure can be detected by various chemical means, including $KMnO_4$, dimethyl sulfate and DEPC. In a model study using the $KMnO_4$ probe,[56] the oligomer containing hairpin structures was briefly exposed to $KMnO_4$ and the modified DNA was isolated by ethanol precipitation. DNA was treated with piperidine and applied directly to a gel (20% polyacrylamide solution). The cleavage products consisted of long DNA fragments together with small nucleotide lengths at the bottom of the gel, which corresponded to the hairpin fragments within the triplet repeat sequences.

Parallel Stranded DNA. Existence of parallel stranded DNA where both strands are oriented in the same 5′–3′ direction has been confirmed by various techniques, including thermodynamic, spectroscopic, and drug binding characteristics as well as chemical modification. The structural discrimination between parallel and antiparallel strands can be conveniently monitored by the modification patterns generated by chemicals ($KMnO_4$, OsO_4, DEPC) in the presence of monovalent and divalent cation concentrations. In one typical experiment,[57] the synthetic parallel and anti-parallel stranded DNA fragments (25 bp) were treated with $KMnO_4$ (80 μg/mL) for 15 min at room temperature. The DNA was obtained by ethanol precipitation and treated with piperidine. The resulting fragments were run on gels and the gel images were analyzed by a densitometric tracer. The autoradiograms indicated that $KMnO_4$ oxidation of parallel stranded DNA was dramatically induced by elevated NaCl concentrations, but not with antiparallel stranded controls. This result indirectly confirmed the destabilizing property of the parallel stranded DNA in comparison with the anti-parallel stranded DNA obtained by previous thermodynamic studies.

3.4. Detection of Aberrant DNA

DNA damage can alter the reactivity of DNA towards the permanganate oxidation reaction. This characteristic was used for designing different assays for specific lesions. Typical examples are the very sensitive assays for detection of thymine dimers[58] and 8-oxoguanine in aberrant DNA.[27]

The Assay for Thymine Dimers. Pyrimidine dimers are lesions leading to skin cancers, generated as a result of [2 + 2] photo-cycloaddition between two adjacent thymine bases on a DNA strand. Various methods have been developed for detection of this lesion, including an enzymatic method (the T4 DNA polymerase does not proceed past the site of dimerization) and HPLC (the experimental sample is compared with synthesized thymine dimer containing fragments). Both methods are cumbersome and error-prone. Due to the desire to improve sensitivity and robustness of this method, $KMnO_4$ was employed as an analytical reagent and showed to be inert with thymine dimers due to lack of the 5,6 double bond. This property led to the development of a convenient and reliable assay to detect thymine dimers in DNA oligomers.[58] Several model synthetic DNA fragments (10 to 22 bp) containing thymine dimers were subjected to $KMnO_4$, followed by piperidine treatment. No strand breaks were observed at the thymine dimers by gel analysis as compared with the control oligomers.

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The Assay for 8-Oxoguanine in DNA. Unlike thymine dimers, the presence of 8-oxoguanine within the DNA sequence enhances the reactivity of $KMnO_4$ at this lesion site. Mechanistic studies on the neighboring base damage induced by $KMnO_4$ oxidation of 8-oxoguanine has recently been studied in single and double stranded DNA.[59] In the single stranded DNA fragment containing a single 8-oxoguanine residue, $KMnO_4$ induced damage at 8-oxoguanine and its neighboring G and T, which was detected by piperidine-induced cleavage as well as enzymatic digestion. After normalization of the cleavage yield, reactivity of common bases with $KMnO_4$ was found to be in the order of G > A > T,C. In double stranded DNA with 8-oxoguanine in one strand, base damage was observed at the bases close to 8-oxoguanine but less efficiently than that of the single stranded DNA. The complexity of the reaction of DNA containing an 8-oxoguanine moiety suggests the new mechanism for damage induction is as follows:[27] $KMnO_4$ reacts with 8-oxo-G, which generates the highly reactive intermediate cation (inducer). The intermediate is produced by double bond oxidation followed by elimination of water. The intermediate cation carries oxidizing ability, which in turn oxidizes the neighboring bases in two migration pathways (electron transfer mediated by stacked bases and intramolecular reaction by direct contact). In an attempt to examine the pathways as well as to characterize the oxidized product, researchers have carried out the $KMnO_4$ oxidation of 5′-*O*-*tert*-butyldimethylsilyl-7,8-dihydro-8-oxo-2′-deoxyguanosine as a model.[59] Three oxidized products were isolated: 5′-*O*-*tert*-butyldimethylsilyl-3,4-didehydro-4,5,7,8-tetrahydro-5-hydroxy-8-oxo-2′-deoxyxanthosines (5.5%), (5-*O*-*tert*-butyldimethylsilyl-2-deoxyribofuranosyl) urea (8%) and (5-*O*-*tert*-butyldimethylsilyl-2-deoxyribofuranosyl)alloxanic acid potassium salt (28%) (Fig. 5). Further studies have also identified three corresponding products derived from the oxidation reaction of 8-oxoadenine.[60] Enhancement of the $KMnO_4$ oxidation was also observed at damaged sites containing 5-hydroxyuracil and 5-hydroxycytosine but their effects at neighboring bases were less profound. These studies have demonstrated the complexity of the reactions and this area of research requires a further study to understand the heterogeneity of the DNA in respond to $KMnO_4$ reactions.

3.5. Detection of Mismatched DNA and Mutations (The CCM Method)

The Chemical Cleavage of Mismatch method (CCM) is one of few methods capable of detecting nearly all single base mismatches.[61] This method was developed in 1988 by Cotton et al. and has been widely used in research and diagnosis of many inherited diseases. The principle of this technology was based on the formation of heteroduplexes derived from equal amounts of wild type and mutant homoduplexes. The mismatched bases (thymine and cytosine) were then subjected to modification reactions with $KMnO_4$ and hydroxylamine respectively.[62] The modified sites were cleaved by piperidine and the resulting fragments were separated and identified by gel-electrophoresis (Fig. 6). The process is time-consuming, as the method requires purification of DNA by ethanol precipitation after each reaction step. To overcome this problem, attempts have successfully been made to attach biotinylated DNA samples onto streptavidin-coated magnetic beads for solid-phase chemical modifica-

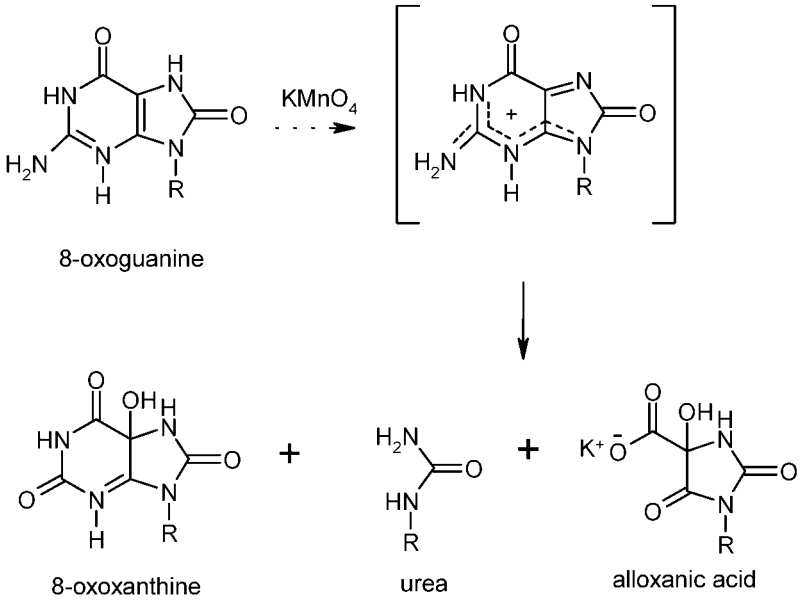

Figure 5. Permanganate oxidation of 8-oxoguanine (R = sugar moiety).

tion and cleavage reactions.[63] The desire to simplify this approach further by reducing the use of the cumbersome biotin dependent assay led to the development of a new solid-phase chemical cleavage of mismatched DNA.[64] The method involves attachment of DNA on to commercially available silica solid supports. The solid-bound DNA remains intact throughout the reactions and the final product is removed from the solid support by treatment with piperidine (1 M) at 90°C. This new version of the CCM method is simple, cost-effective and highly accurate by improving the quality of the cleavage fragments on sequencing gel traces.

Several small chemical molecules have been developed for detection of single base mismatches and, thus, mutations.[28] Each method has its merits although some appear to have significant limitations such as a requirement of an attachment of tagging systems for detection (biotin-carbodiimide complex), application in only multiple mismatches or DNA bulges only (DEPC), or non-universality for all mismatch types (metal complexes), etc.

Chemical cleavage of mismatch has been widely used as a diagnostic tool for detection of mutations in many inherited diseases.[4] Recent studies indicated that all four bases (T, C, A and G) at the mismatched site were susceptible towards reaction with $KMnO_4$. Therefore, it was suggested to be used as a unique probe for detection of all possible base changes in DNA fragments (up to 2 kb).[63]

3.6. Spectroscopic Assays

The permanganate oxidation reaction releases the soluble by-product MnO_2, which gives strong absorption at 420 nm. This wavelength has been employed for

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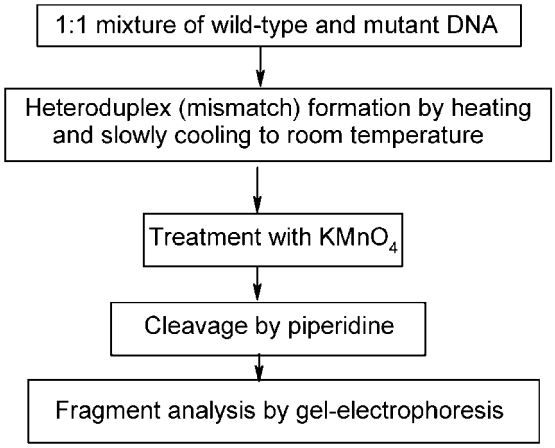

Figure 6. The chemical cleavage of mismatch (CCM) method.

quantitative measurement of the oxidation level of reactions, as well as the rates of the reaction.[19] Recently, reaction of $KMnO_4$ with naltrexone was reported to be chemiluminescent in sulfuric acid medium.[65] The emission was produced immediately after the injection of chemicals and remained unchanged despite further increasing acidic concentrations. The reaction was further developed into a chemiluminescent assay, using a luminometer for quantitation of drug levels based on the calibration curve with high a correlation coefficient (> 0.99). Various chemicals such as phenytoin and carbamazepine (antiepileptic drugs), when subjected to $KMnO_4$ oxidation, produced the corresponding oxidized products with strong absorption at their particular wavelengths (247 nm for phenytoin; 247 nm and 372 nm for carbamazepin). Based on these characteristics, a quantitative spectroscopic method for simultaneous analysis of two chemicals in a single sample has been developed.[66]

4. COMPARATIVE STUDIES WITH OTHER CHEMICALS

Several chemicals have been employed as modifying agents for nucleic acids. Each has its merits and seems to be suitable for specific applications. The choice of chemical depends on two major factors: the degree of DNA unwinding and chemical properties of the modifying agents (e.g., reactivity, solubility, size of molecule, electric charge, etc.,). Enzymes, binding proteins (receptors) and chemicals (peptide and small chemicals) can induce large conformational changes when they come into contact with DNA. The resulting open complex of DNA enhances chemical activities of various modifying agents such as $KMnO_4$, OsO_4, and DMS. However, both OsO_4 and DMS were later found to be less valuable due to toxicity (OsO_4) or low reactivity (DMS) in the footprinting assay.[6] Similarly, $KMnO_4$ and DEPC have been widely used for detection of loop structures of DNA, like multiple base mismatches, deletions and insertions.[7] In these cases, the DNA conformation displays a large degree of base exposure for chemical reactions to take place. When the process involves low destacking events such as a single base mismatch, DEPC was reported to be

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unsuitable probably due to the steric effect and/or the neutrality of this molecule. Detection of mismatched bases requires chemicals with exceptional reactivities such as $KMnO_4$, hydroxylamine and carbodiimide. Both $KMnO_4$ and hydroxylamine were employed to modify thymine and cytosine respectively in CCM while the carbodiimide application is somewhat cumbersome as it requires complex synthesis of a fluorescent moiety attached to the molecule for detection purposes.[67] Recently, $KMnO_4$ was used as a self-detectable chemical for quantitative analysis of small molecules (drug and nucleotide bases), based on its chemiluminescent reactions. The mechanism of the chemiluminescent reaction is unknown but it is believed that $KMnO_4$ is reduced to an electronically excited manganese complex, which then emits a photon.

Out of the strongest oxidizing agents, $KMnO_4$ is preferentially selected for biological assays. Nitrogen oxide (NO) selectively oxidizes adenine, guanine and cytosine to afford hypoxanthine, xanthine and uracil respectively,[68] and ozone (O_3) preferentially attacks the double bond of pyrimidine bases to give the corresponding hydantoin.[69] Both chemicals have limited application due to the gas phase of the reactants. Hydrogen peroxide and *m*-chloroperbenzoic acid were used for preferential modification of purine bases at the N1 and N7 positions, respectively, but no application has been reported so far.[70]

5. CONCLUSION

As we have discussed in this review, $KMnO_4$ is a useful chemical probe for studying DNA structural changes induced by various molecules (enzyme, hormone receptor, intercalator, drug-like molecules, etc.). All applications are based on the site-selective oxidation reaction of $KMnO_4$ with nucleotide bases at a specific site of the DNA sequence. Detection of modified bases in the DNA molecule requires cleavage with piperidine and the resulting fragments are subsequently analyzed by gel electrophoresis. While the method has proven its usefulness in both in vitro and in vivo conditions it suffers from the following drawbacks:

(i) DNA conformational changes are not fully elucidated as the results are merely based on fragment analysis by gel-electrophoresis.
(ii) The permanganate oxidation reaction of DNA requires extensive optimization studies due to lack of current kinetic and thermodynamic information associated with this type of reaction on DNA.
(iii) Although $KMnO_4$ has been successfully used in both in vivo and in vitro conditions, diffusion of the molecule through the cell membrane and the interference effects of cellular metabolites have not been studied.
(iv) Toxic piperidine is involved in the process and its residue might not be suitable for further biological assays unless the test sample is purified.
(v) The procedure is laborious due to the time-consuming gel electrophoresis and DNA purification after each reaction.

Recently, some approaches have emerged in an attempt to avoid the cumbersome detection steps (piperidine/gel electrophoresis). Use of electrochemical detectors was developed for analysis of oxidized nucleosides.[71] Polyclonal antibodies

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raised against DNA modified with $OsO_4$was successfully reported.[72] In principle, this approach can be applied for the detection of DNA oxidized with $KMnO_4$ as both reagents are chemically equivalent and the oxidized products are the same.

Inputs from the research interface between chemistry and biology are required to deal with the above questions together with those described in the section 2 (mechanism of the reaction) as they highlight the bottleneck for expanding the chemical applications of $KMnO_4$ in medical research and the diagnostic industry in terms of high-throughput applications.

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