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Liquid chromatography- mass spectrometry for analysis of DNA damages induced by environmental exposure

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ABSTRACT

Long-term exposures to environmental toxicants are causative factors for human health effects. DNA damages are of great interest in that they can serve as the potential biomarkers of environmental exposure and mutations. Qualitative and quantitative detection of their level in genomic DNA, are helpful for the understanding of the generation, transformation, repair and fate of DNA damages and for elucidating the mechanism underlying carcinogenesis. Methodologies based on liquid chromatography-mass spectrometry (LC-MS) have shown unique advantages in accurate and highly sensitive analysis of these structurally modified DNA damages. The present review summarizes our current knowledge on LC-MS analysis of genetic DNA damages, with special emphasis on structurally chemical DNA modifications induced by environmental exposure. Advances in LC-MS analysis and monitoring of oxidative DNA lesion, DNA adducts induced by heavy metals, persistent organic pollutants, bisphenols, fine particulate matters, aldehydes and halobenzoquinones are comprehensively summarized.

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1. Introduction

Some of environment pollutants may cause various DNA damages [1,2]. Only a few environmental chemicals are inherently reactive to DNA, while more hazardous chemicals need first to be metabolically activated to electrophilic intermediates, or induce reactive oxidative species (ROS) before reaction with DNA. These metabolic intermediates are more active to attack DNA skeleton, resulting in nucleobase damages, including DNA strands breakage, intra-and inter-strand DNA cross-links, chemically modified nucleobases [2–4].

Of note, almost all of normal mammalian cells possess extensive and complicated DNA repair systems, which are responsible for removing these nucleobase damages, maintaining the normal physiological activities. However, if DNA damages escape from repair, they may cause base mispairing during DNA replication which can be converted into permanent mutations. Accumulation of mutations in genes controlling cell growth, proliferation, programmed cell death, and cell differentiation is likely to cause cancer [5].

In the viewpoint of chemistry of DNA damages, structural DNA modifications are formed on the nucleobase or phosphate moieties of DNA backbone [6–9]. Usually, the nucleobase damages are considered to have direct relationship with chemical carcinogenesis, thus being more frequently studied than phosphate group modifications [7]. Typical nucleobase damages includes the oxidation, deamination, alkylation, and cross-linking occurred at a variety of nucleobase sites, for instance, the N-7, O-6, C-8, and N-2 of guanine, the N-1, N-3, and N-7 of adenine, the O-2 and O-4 of thymine, and the O-2 and N-4 of cytosine [8,9]. Fig. 1 shows the reactive sites on nucleobases on which adducts and oxidative damage are preferably formed. A part of these DNA modifications are hereditable in cell division, which means the injuries associated with DNA damage have lasting impacts, even environmental exposure is eliminated.

The present review summarizes our current knowledge on the LC-MS analysis of genetic DNA damages, with special emphasis on structurally chemical DNA modifications induced by environmental exposure.

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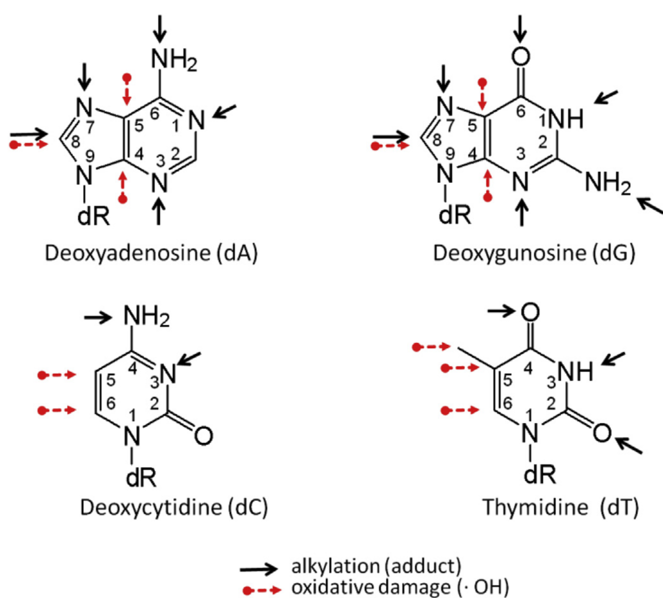

Fig. 1. Reactive sites on nucleobases (nucleotides) where damages are preferably formed.

2. LC-MS strategies for structurally modified DNA analysis

While multiple methodologies have been proposed for analysis of DNA damage in biological samples, including comet assay, ^{32}P postlabeling and immune-base assays [10–12], LC-MS strategies generally have superior selectivity, sensitivity, accuracy, and reproducibility [13]. Significant advances have been made in the chemical analysis of DNA damage in recent years. These include the enhancement of detection sensitivity, development of potent DNA pretreatment strategies, application of high-resolution mass analyzers (i.e. Orbitrap and time-of-flight), DNA adductomics and screening of DNA damages caused by new emerging contaminants [4,9].

The content of damaged nucleotides is usually very low (0.1–100 lesions per 10^8 nts), but the changes occurring at low content levels make sense to certain biological implications. A serious challenge is how to detect ultra-low level of DNA modifications in the presence of a large number of normal nucleosides. Therefore, the development of ultra-sensitive mass spectrometry-based detection technology is of great significance for accurately assessing the biological functions and health risks. Essentially, 2′-deoxyribonucleosides are polar compounds, but their MS responses (i.e. sensitivity) is not very high adequately. Many efforts have been devoted to increasing the sensitivity of LC-MS for nucleoside detection.

Firstly, attempts have been made in chemically modifying 2′-deoxyribonucleosides with labeling reagents for enhancing the sensitivity of MS analysis of DNA modifications. Aryldiazomethane derivatives as potent reagents for site specific labeling of nucleic acids at phosphate moieties were firstly proposed by Bourget et al. [14]. Inspired by this attempt, Yuan et al. showed the selective derivatization of cytosine moieties with 2-bromo-1-(4-dimethylamino-phenyl)-ethanone (BDAPE) for the simultaneous determination of modified cytosines in genomic DNA [15]. This derivatization greatly improved the LC separation and dramatically increased MS detection sensitivities of these cytosine modifications. They further synthesized 8-(diazomethyl) quinoline (8-DMQ) from quinoline-8-carbaldehyde by referring to Bourget's protocol [14]. This synthesized 8-DMQ can selectively and high efficiently react with the terminal phosphate group under mild condition to label NTPs. By combining this chemical labeling method with LC-ESI-MS analysis, NTPs could be sensitively detected with the detection limits improved by 56–137 folds [16]. They detected 12 types of modified NTPs in the mammalian cells and tissues, including 5-methylcytidine 5′-triphosphate, 5-methyl-2′-deoxycytidine, 5′-triphosphate, 5-hmCTP, 5-hmdCTP, 7-meGTP, 2′-O-ATP, 2′-O-GTP, N^1-meATP, and N^6-meATP. Taking advantage of this method, they showed that the content of the majority of the modified NTPs significantly decreased in human hepatocellular carcinoma (HCC) tissues compared to tumor-adjacent normal tissues.

Our strategy is to develop an ammonium bicarbonate (NH_4HCO_3)-enhanced LC-MS/MS method for sensitive and accurate quantification of DNA modifications [17]. Ammonium bicarbonate is thermally unstable and easily degraded into carbon dioxide and ammonia in the heated gas phase. In principle, ammonium bicarbonate as an additive in the mobile phase improves the protonation of some modified 2′- deoxynucleotides, and also inhibits the formation of the metal -2′ -deoxynucleoside complex, which worsens the MS signal during electro-spray ionization, and significantly enhances its MS/MS responses. Typically, it can improve the sensitivity of their MS detection up to an order of magnitude. Taking advantage of this method, we have shown a number of modified nucleosides to be enhanced for MS detection, including 5hmdC, 5fdC, acrolein- and crotonaldehyde-reacted dG adduct [18].

3. LC-MS analysis DNA damages induced by environmental exposure

3.1. Oxidative DNA damages

Formation of oxidatively damaged nucleobases such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG, also known as 8-OHdG) has been associated with aging and carcinogenesis. Besides 8-oxodG, other major oxidative modifications (Fig. 2) including 7,8-dihydro-8-oxoadenine (8-oxoA), 2-Hydroxyadenine (2-OHA), 2,6-diamino-4-hydroxy- 5 -formamidopyrimidine (Fapy-G), and 4,6-Diamino-5-formamidopyrimidine (Fapy-A), 5,6-dihydroxy-5,6-dihydrocytosine, 5,6-dihydroxy-5,6-dihydrothymine, 5-hydroxymethyluracil, and 5-formyluracil, have been identified in the mammalian genome [19]. The cellular responses to such oxidative damage involve several processes, for instance, DNA repair, cell-cycle arrest and apoptosis. Exposure to carcinogen pollutants, such as heavy metals, PAHs, halogenated quinones, bisphenols and other persistent toxic substances causes oxidative DNA damages.

3.1.1. Heavy metals

Carcinogenic transition metals, e.g., cadmium (Cd), chromium (Cr), and nickel (Ni), and the metalloid arsenic (As), can bind to the nucleus in cells and cause induced promutagenic lesions, including DNA strand breaks, nucleobase modifications, rearrangement and depurination (AP sites), intra- and inter-molecular crosslinking of DNA and proteins [20]. Potential mechanisms include the promotion of ROS and other free radicals, which are produced by metal-catalyzed redox reactions of oxygen, hydrogen peroxide, lipid peroxides and other substances. One of the most widely-accepted mechanisms for ·OH production is through the transition metal-catalyzed Fenton or Fenton-like reactions. Metal ions interacting with H_2O_2 produce not only free ·OH radicals but also other strong oxidants, such as singlet oxygen, and metal-centered -oxo and -peroxo species, all capable of damaging DNA and proteins. Kordas et al. [21]examined the cross-sectional association between urinary concentrations of As, Cd and lead (Pb) (by HPLC-ICP-MS) and

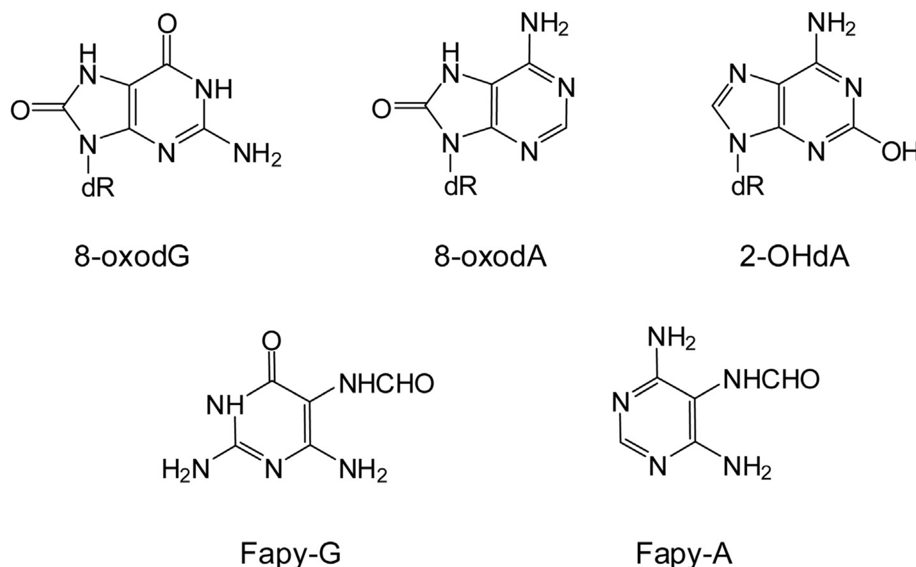

Fig. 2. Structures of the representative oxidative nucleobase lesions. 7,8-dihydro-8-oxoguanine (8-oxoG), 7,8-dihydro-8-oxoadenine (8-oxoA), 2-Hydroxyadenine (2-OHA), 2,6-diamino-4-hydroxy- 5 -formamidopyrimidine (Fapy-G), and 4,6-Diamino-5-formamidopyrimidine (Fapy-A).

urinary oxidative markers 8-oxodG and F2-8α isoprostane (by ELISA assay) in 211 children aged 6–8 years living in Montevideo, Uruguay. Log2-transformed urinary concentrations of As, Cd and Pb was positively associated with 8-oxodG concentrations. Even at low-level, As exposure is associated with detectable oxidative damage to the DNA. The cytotoxicity and genotoxicity of Cadmium, Lead, and Arsenic on the Sertoli cell and HT-22 Hippocampal cell lines, together with the mechanisms of damage involved, were summarized by Ramos-Treviño et al. [22]. Of note, exposure to carcinogenic transition metals also causes epigenetic DNA alternations as reviewed by Yuan et al. [23]. Our recent study revealed that nickel(II) ion can directly displace the cofactor iron(II) of Tet dioxygenases and inhibit Tet-mediated 5 mC oxidation *in vitro* [24]. We found that nickel exposure can reduce DNA demethylation and potentially change the methylation status of specific genes in somatic (HEK293T and MRC-5) and mouse ES cells, but not global DNA hypermethylation. Our study provided a novel mechanistic insight into changes in nickel-induced DNA methylation.

3.1.2. Persistent organic pollutants

Both PAHs, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been found to cause oxidative DNA damages via ROS generation [25–30]. B[a]P derivatives have been reported to have the capacity to enter redox cycles and induce the production of ROS, thereby resulting in oxidative DNA damages [25]. Gao et al. [27] investigated the relationship between 2,2,4,4-tetrabromodiphenyl ether (BDE-47) and oxidative DNA damage as well as the mode of interaction between BDE-47 and 2,2,4,4,5,5-hexachlorobiphenyl (PCB153) by incubating SH-SY5Y cells. They found that DNA strand breakage and 8-oxodG levels were significantly increased in the 10 μM PBDE-47, 5 μM PBDE-47 + PCB153, and 10 μM BDE-47 + PCB153 groups compared with the control ($p < 0.05$). Results indicate that BDE-47 induced oxidative DNA damage and that PBDE-47 combined with PCB153 may increase such effects in SH-SY5Y cells *in vitro*. *In vivo* experiment was conducted by Mutlu et al. [28]. They examined 8 oxidative products in female Sprague-Dawley (SD) rats by LC-MS/MS analysis, including 8-oxo-dG, 1,N^6-ethenodeoxyadenosine (1,N^6-εdA), N^2,3-ethenoguanine (N^2,3-εG), 1,N^2-ethenodeoxyguanosine (1,N^2-εdG), as well as malondialdehyde (M1dG), acrolein (AcrdG), crotonaldehyde (CrdG), and 4-hydroxynonenal-derived dG adducts (HNEdG). Increased hepatic oxidative DNA adducts following exposure to PCB 126, PCB 153, or the binary mixture shows that the elevated level of DNA damage may play an essential role in hepatic toxicity and carcinogenesis in female SD rats. Similarly, PBDEs such as BDE47, BDE209, and BDE153, also have been found to be capable of causing oxidative DNA damage both *in vitro* and *in vivo* [29,30].

Polychlorinated dibenzo-dioxins and polychlorinated dibenzofurans (PCDD/Fs) also have been reported to induce ROS and oxidative stress. Zhang et al. [31] examined urinary 8-oxodG and 8-iso-prostaglandin-F2α (8-isoPGF2α) of 602 participants to explore oxidatively damage to DNA and lipids. They found that PCDD/Fs exposure and urinary oxidative stress biomarkers of workers were all higher than those of the reference group.

3.1.3. Halogenated quinones

Halobenzoquinones (HBQs) have been identified as new types of halogenated disinfection byproducts (DBPs) widely distributed in disinfected drinking water and swimming pool water [32,33]. The extensive existence of HBQs in drinking water may pose various adverse effects to human healthy safety. Because HBQs are oxidants and belong to electrophilic species, they can not only form adducts through alkylation of DNA and/or proteins, but also induce oxidative damages to DNA due to formation of ROS, including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical [33]. Li et al. reviewed the chemical and toxicological characteristics of HBQs and concluded that the *in vitro* evidence of oxidative stress-induced DNA damage might be responsible for their genotoxicity [34]. They found that HBQs exposure could induce an excessive increase in ROS and formation of 8-oxodG and carbonyls of proteins in both human bladder cancer (T24) and Chinese Hamster Ovary (CHO–K1) cells [35]. These *in vitro* evidences demonstrated HBQs are both cytotoxic and genotoxic.

Our previous work demonstrated that HBQs/H_2O_2 can effectively oxidize DNA through transition metal ions-independent mechanisms [36]. TCBQ and H_2O_2 induce oxidative damage to both 2′-deoxyguanosine and ctDNA *in vitro*. The oxidative potency of HBQs to ctDNA is even higher than that of the classic iron-mediated Fenton reaction. More recently, we investigated the levels of 8-oxodG in genomic DNA of bladder cancer (T24) cells

treated with HBQs, using HPLC-MS/MS with MRM mode. It was found all HBQs significantly induced the formation of 8-oxodG in a dose-dependent manner; and the oxidative potency of HBQs to T24 cells was in an order of 2,5-DCBQ ≈ 2,6-DCBQ > TCBQ > TBBQ. This is quite a different tendency in 8-oxodG formation from the *in vitro* experiment by reacting HBQs/H_2O_2 with ct-DNA [37].

3.1.4. Bisphenols

Bisphenol A and its halogenated analogues (BPs) have been widely used in industrial production in particular as plasticizers and flame retardants [38]. BPs have become the focus of intense public scrutiny following concerns about its association with human diseases such as reproductive disorders, obesity, diabetes and cancer [39,40]. Concern about BPA exposure is often linked to its estrogenic properties, but recent studies linked BPs with DNA lesions, either genomic damages or epigenetic modifications [41]. Oxidative stress appeared to play a role in the BPA-induced proliferation and DNA damage, as evidenced by a partial reversal of both processes upon pretreatment with an antioxidant, *N*-acetylcysteine [42]. Barbonetti et al. examined the effects of *in vitro* BPA exposure on human sperm integrity and found that 300 μM of BPA could cause significant DNA oxidative damage to sperm by formation of 8-oxodG [43]. Besides the 8-oxodG, the oxidative stress induced by bisphenols can also create stable base lesions and abasic sites in genomic DNA. By integrating LC-MS analysis and molecular toxicology technologies, Zhao et al. [44] examined the toxicity of tetrabromo bisphenol A (TBBPA) and tetrachloro bisphenol A (TCBPA) on cell viability, ROS, and metabolic alterations. They found low-concentration (0–10 μM) exposure of TCBPA/TBBPA promotes cell proliferation and activates metabolism of glycolysis and amino acids, whereas high-concentration (10–50 μM) exposure of TCBPA/TBBPA promotes ROS generation *via* the down-regulating glutathione biosynthesis and up-regulating nucleotides metabolism. Five major oxidative metabolites (adenosine, adenine, hypoxanthine, xanthine and guanine) and mRNA levels of xanthine oxidase were found to be up-regulated in the BPs-treated group at 50 μM, which is helpful for better understanding the cytotoxicity of TCBPA and TBBPA by regulating the specific metabolic pathways.

3.1.5. Fine particulate matters

As a new emerging pollutant, particulate matter with the aerodynamic equivalent diameter ≤ 2.5 μm (PM2.5) has attracted extensively attention in recent years. Emerging evidences indicated that PM2.5 had significant impact on air quality, climate, and human health especially concerning respiratory and cardiovascular health [45–48].

The adverse effects that PM2.5 exerts on human health are quite complex. Chemicals in PM2.5 are at different levels and there are various signaling pathways that are implicated in the mechanisms of DNA damage [49]. Abbas et al. investigated the cytotoxicity, genotoxicity and pro-inflammatory response of the human bronchial epithelial BEAS-2B cells exposed by organic extractable matter components from ambient PM2.5, revealing that the promotion of oxidative damage takes essential role in explaining the cytotoxicity and acute toxicity [50].

Iron (Fe) was found as the most abundant metal in PM, with significant bioaccessibility [51]. Smaller-sized PM2.5 particles carry more Fe(III), which facilitates the generation of hydroxyl radicals. In this vein, PM2.5 showed more oxidative damage than PM10, which implies that PM2.5 particles are more toxic and have higher oxidant-generating capacity than PM10. PAHs including B[a]P, chrysene(Chr) and indeno[1,2,3-cd]pyrene (IcdP) were determined in PM2.5 and found to have significantly positive correlations with the DNA damage rates. Additionally, the correlation between B[a]P equivalent concentrations and DNA damage rates suggested that synergistic reaction could affect the toxicity of PAHs [52]. A case study further illustrated this impact of PM2.5 on human health, in which the influence of PM2.5 exposure on biomarkers of oxidative stress was examined with urine samples from individuals travelling from Germany to China [53]. LC-MS/MS methods were used for the determination of biomarkers including 8-oxodG, malondialdehyde, F2α-isoprostanes and hydroxylated PAHs, and correlations were found between exposure and these oxidative stress biomarkers. Interestingly, the researchers claimed that travellers might be ideal models for study of PM pollution-induced acute health effects [53].

3.2. DNA adducts

3.2.1. Alkylated DNA adducts

Genotoxic chemicals can covalently bind with the reactive sites in DNA and cause a diverse array of DNA adducts. The most extensively studied are alkylated DNA adducts, which generated through the covalent reactions between nucleobases and alkylating chemicals, including methylating and ethylating agents, aromatic compounds, nitrosamines, aflatoxins, mustards, and haloalkanes. Studies of these DNA alkylation-inducing chemicals uncovered most ring nitrogen atoms as well as exocyclic oxygen and nitrogen atoms of nucleobases as targets for alkylation. Generally, C8, N7, N3, N2, N1, and O6 positions of guanine, the N7, N6, N3, and N1 positions of adenine, the N3, N4, and O2 positions of cytosine, and the N3, O2, and O4 positions of thymine, are susceptible to these alkylating chemicals. In addition, etheno derivatives of guanine, adenine, and cytosine can arise from either the metabolic activation of exogenous chemicals (e.g. vinyl chloride or ethyl carbamate) or endogenous lipid peroxidation [54]. Their targeting sites are almost all localized between the exocyclic N and one of its neighboring ring N atoms, which cause the formation of 1,N^2- and 3,N^2-ethenoguanine (εGua), 1,N^6-ethenoadenine (εAde), and 3,N^4-ethenocytosine (εCyt) [55].

Among the alkylated DNA lesions, O^2-alkylthymidines (O^2-alkyldT) are considered to be resistant to repair and thus persist in mammalian systems [56,57]. Zhai et al. [56] synthesized oligodeoxyribonucleotides harboring seven O^2-alkyldT adducts at a defined site then examined the impact of these lesions on DNA replication in *E. coli* cells. Alkylated DNA lesions were identified and quantitatively detected by LC-MS/MS on an LTQ linear ion trap MS instrument. It was found that the replication bypass efficiencies of O^2-alkyldT adducts decreased with the length of the alkyl group, which directed promiscuous nucleotide misincorporation in *E. coli* cells. By the use of stable isotope dilution nanoLC–nESI MS/MS, Chen et al. [57] detected the levels of O^2-ethylthymidine in human leukocyte DNA, and found that O^2-alkyldT levels in smokers were significantly higher than that in nonsmokers.

Recently, Gelatt et al. [58] revealed that 1-methoxy-3-indolylmethyl (1-MIM) glucosinolate is mutagenic and forms DNA adducts *in vivo* in mouse experiment. Administering mice with 1-MIM glucosinolate for 1–14 days, N^2-(1-MIM)-dG and N^6-(1-MIM)-dA adducts in six tissues were determined using UPLC–MS/MS with isotopically labeled internal standards. Result showed that, during the 14-day recovery period, N^2-(1-MIM)-dG in liver, kidney, lung, jejunum, cecum and colon decreased to 52, 41, 59, 11, 7 and 2%, respectively.

3.2.2. Aldehyde-DNA adducts

Formaldehyde and acetaldehyde are both mutagenic and genotoxic, which are classified as Class 1 and Class 2B carcinogens by International Agency for Research on Cancer (IARC). Formaldehyde is an active electrophilic molecule that able to attack nucleophilic dG and dA in DNA to form N^2-hydroxymethyl-deoxyguanosine

(2 hmdG) and N^6-hydroxymethyl-deoxyadenosine (6 hmdA) (Fig. 3), respectively. These two products of covalent reaction are both unstable when are present in the formation of nucleoside. In vivo, 2 hmdG and 6 hmA can be reduced to stable N^2-methyl-deoxyguanosine (2mdG) and N^6-methyl-deoxyadenosine (6mdA) by endogenous reducing agents with a low conversion of 6%–8%. These reduction processes can be implemented by introducing the chemical reduction such as sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$) *in vitro*. The conversion rate is obviously increased up to approximately 50%–80% [59]. Similarly, acetaldehyde could react with the exocyclic amino group of 2′-deoxyguanosine (dG) in DNA to form N^2-ethylidene-deoxyguanosine (2etidG) primarily, which is an unstable Schiff base, but can be stabilized by reduction with glutathione (GSH) and L-ascorbic acid (Vitamin C) *in vivo* or $NaBH_3CN/NaBH_4$ *in vitro*, forming stable N^2-ethyl-deoxyguanosine (2ethdG) [60,61]. The 2etidG adduct is considered to be chemically stable in DNA, but quickly breaks down at single deoxynucleoside level with a half-life of 5 min at 37 °C. The abundance of 2 hmdG, 6 hmdA and 2etidG in cells could be determined by the detection of corresponding 2mdG, 6mdA and 2ethdG after chemical reduction of DNA during isolation and/or enzymatic hydrolysis. For this reason, these unstable DNA adducts were indirectly detected in DNA from various samples including cultured cells and tissues [62].

Acrolein and crotonaldehyde are two widespread and toxic α,β-unsaturated aldehydes, found in tobacco smock, automobile exhaust, combustion of fossil fuel and other pollution emissions. Acrolein can react with the exocyclic amino group of dG in DNA, forming two structural isomer adducts of α-hydroxy-1,N^2-propano-deoxyguanosine (α-AcrdG) and γ-hydroxy-1,N^2-propano-deoxyguanosine (γ-AcrdG) (Table 1). Meanwhile, α-AcrdG has two stereoisomers in dynamic equilibrium. In addition to AcrdG, our study demonstrated that acrolein adducted deoxyadenosine (AcrdA) and deoxycytidine (AcrdC) can also be formed [17]. The reaction of crotonaldehyde with dG in DNA can produce (*6S,8S*) and (*6R,8R*) diastereomers of α-Methyl-γ-hydroxy-1,N^2-propano-deoxyguanosine (CrodG). CrodG adducts are also products of the reaction of two-molecule acetaldehyde with one-molecule dG. Interestingly, each isomer is discrepant in genotoxicity, α-AcrdG has higher mutagenicity than γ-AcrdG and (*6S,8S*)ProdG can cause more frequently miscoding than (*6R,8R*)ProdG (Fig. 3) [63,64].

We have developed an NH_4HCO_3-enhanced UHPLC-MS/MS method for simultaneous quantification of isomeric AcrdG, AcrdA and AcrdC adducts in combination with stable isotope dilution [17]. By employing the developed method, trace amounts of Acr-DNA adduct are quantified in cultured A549 cells without acrolein treatment and human leukocytes. We applied this method to detect diastereomers of CrodG adducts (with frequency of 2.4–3.5 adducts per 10^8 nucleotides) in MRC5 cells treated with acetaldehyde or crotonaldehyde [63]. The levels of CrodG adducts in human MRC5 were found to be apparently elevated by the exposure of acetaldehyde or crotonaldehyde in a dose-dependent manner. Then two diastereomers of CrodG in human urine were accurately quantified at fmol/L level, providing evidence supporting the presence of CrodG adducts in human urine [64].

3.2.3. Bulky aromatic adducts

The bulky DNA adducts are formed by the covalent binding of those chemical carcinogens with large size, such as PAHs and aromatic amines, to various sites on DNA bases. They are usually highly mutagenic, as exemplified by the PAH-DNA adducts [65].

Benzo[a]pyrene can be stereoselectively metabolized *in vivo* to a reactive metabolite, (+)-*anti*-benzo[a]pyrene diol epoxide (*anti*-BPDE), which may predominantly bind to deoxyguanosine (dG) and cause BPDE-dG adducts. We have developed an improved LC-MS method for simultaneous analysis of four stereoisomeric BPDE–N^2-dG adducts, *trans-(+)*, *trans*-(−), *cis*-(+) *and cis*-(−) -BPDE-dG (Fig. 4), in short oligonucleotides using, providing a

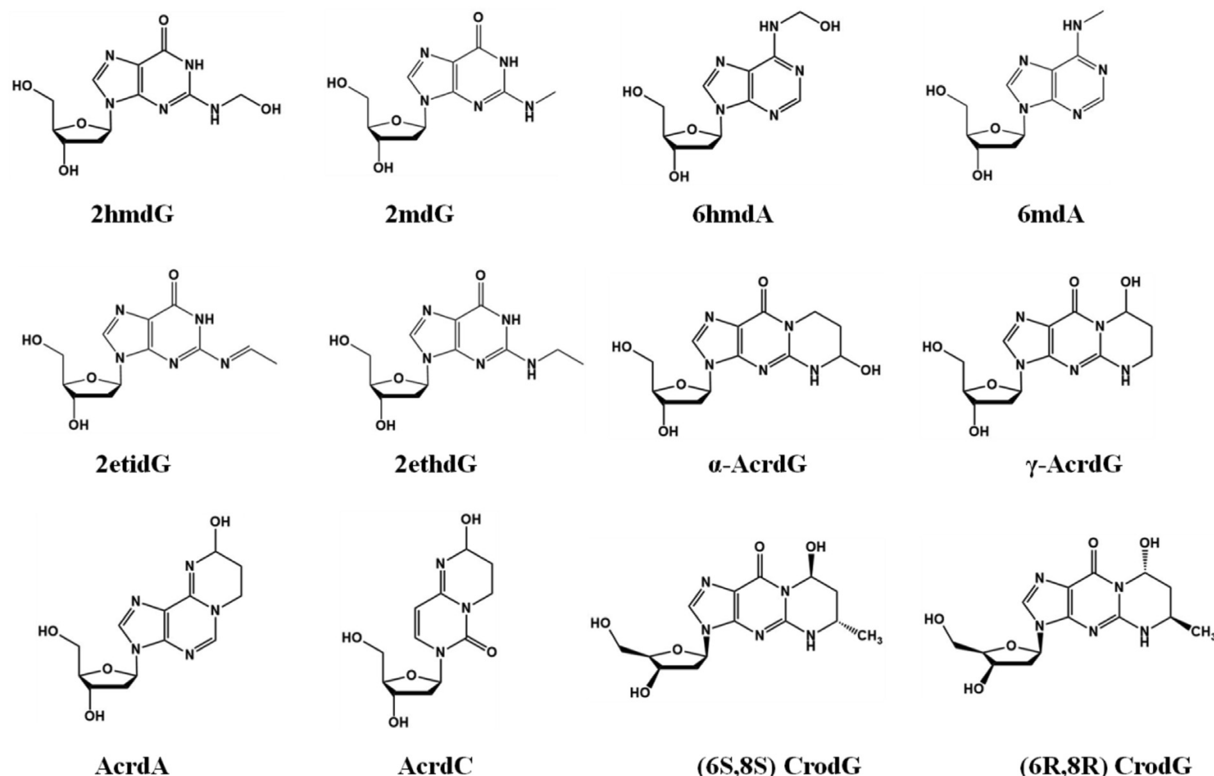

Fig. 3. Structures of the representative DNA adducts induced by formaldehyde, acetaldehyde, acrolein and crotonaldehyde.

Table 1
Chemical structure of DNA adducts induced by aldehydes exposure.

Aldehyde	Molecular formula	Structural formula	Adducts	Refs
Formaldehyde	CH_2O		N^2-hydroxymethyl-dG N^6-hydroxymethyl-dA	59
Acetaldehyde	C_2H_4O		N^2- ethylidene-dG R-/S-α-methyl-γ-hydroxy-1,N^2-propano-dG	60,61
Acrolein	C_3H_4O		α-/γ-hydroxy-1,N^2-propano-dG	17
Crotonaldehyde	C_4H_6O		R-/S-α-methyl-γ-hydroxy-1,N^2-propano-dG	63,64

selection strategy of stationary phase for analysis and separation of PAH–DNA adducts [66]. Then, we enhanced the detection sensitivity and accuracy by employing UPLC-MS/MS with a MRM mode. The developed method displays a low limit of detection of <0.7 fmol (S/N = 3) for the four stereoisomers of anti-BPDE–N^2-dG, allow for the study of stereoselectivity of metabolic activation of B[a]P in human lung A549 cells [67].

Guo et al. [68] employed an LC-MS/MS method to detect and quantity BPDE-dG adducts present in 42 normal human umbilical cord blood samples and 42 birth defect cases. As a result, there is no significant difference in the level of BPDE-dG formation between the normal and birth defect groups. Villalta et al. [69] developed an LC-ESI-MS/MS method employing high-resolution/accurate mass analysis for detecting ultralow levels of BPDE-dG adducts in human lung cells. The developed methodology had a limit of detection (LOD) of 1 amol of BPDE-N^2-dG on-column, corresponding to 1 BPDE-N^2-dG adduct per 10^{11} nucleotides (1 adduct per 10 human lung cells) using 40 μg of DNA. Up to date, this is probably one of the most sensitive DNA adduct quantitation method yet reported, as the authors claimed, exceeding the sensitivity of the ^{32}P-postlabeling assay (~1 adduct per 10^{10} nucleotides).

As a contrast to PAHs, there are a limited number of reports concerning the detection of DNA damages caused by heterocyclic aromatic amine (HAA) carcinogens in biological samples. Highlighted works in recent years on HAA-DNA adducts analysis and the related biological effects were mainly contributed by Turesky's research group. They have developed online DNA adduct enrichment and UPLC-ESI- multistage ion trap MS (UPLC-ESI-IT-MS^n) to quantify and characterize DNA adducts of 4-ABP and HAAs in rodent and human tissues and human saliva [70,71], successfully employed tissues to screen for DNA adducts of aristolochic acid-I and PhIP in paired fresh-frozen (FR) and formalin fixed paraffin embedded (FFPE) human renal and prostate tissues by high-resolution accurate mass (HRAM) nanoUPLC-ESI-Orbitrap-MS/MS [72]. The method has a limit of quantification (LOQ) of 1–2 adducts per 10^9 nucleotides, when employing 10–20 μg DNA. More recently, they proposed an untargeted, unbiased data-independent screening method, termed wide-SIM/MS/MS, for HAAs induced adductomics study [73], which can screen a wide-range of unknown HAAs-DNA adducts in human tissues.

3.2.4. Endocrine disrupting chemicals (EDCs) and bisphenol chemicals (BPs)

An increasing broad spectrum of compounds, both natural and synthetic can be considered EDCs, such as pesticides, plasticizers, polycyclic aromatic hydrocarbons and hormones. Evidence exists

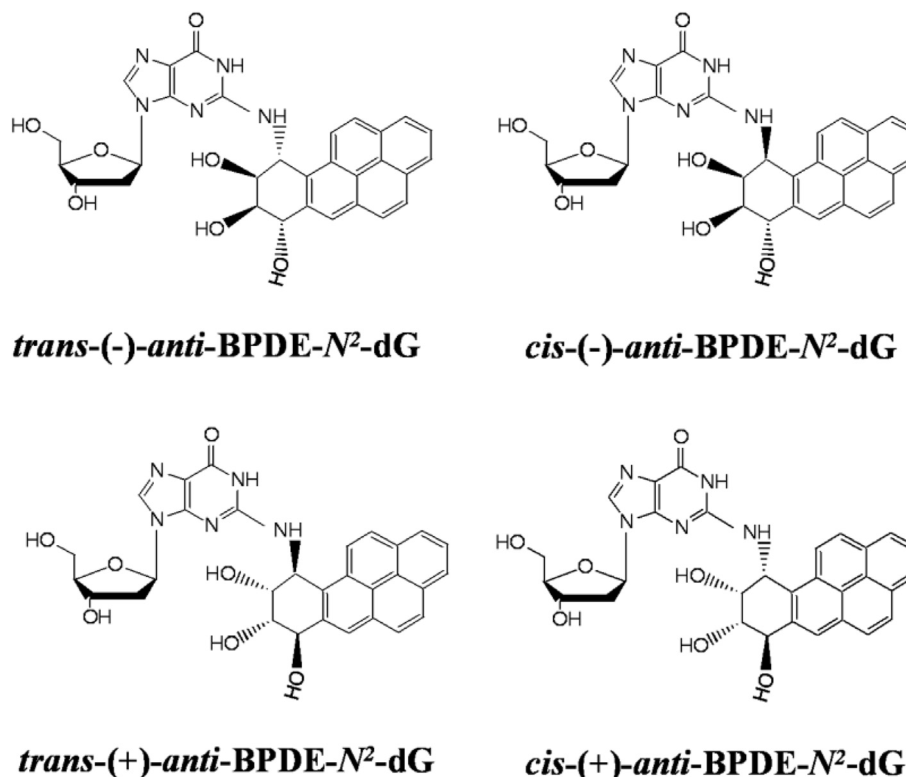

Fig. 4. Structures of stereoisomers of *anti*-BPDE-N^2-dG adducts.

that raises concern about genotoxic effects induced by estrogen, including the oxidative stress caused by estrogen-derived oxidants, and DNA adducts formed by estrogen metabolites and estrogen-induced chromosomal aberration [74].

Human exposures to bisphenol chemicals have attracted considerable attentions on global health. Cytotoxicity analysis showed that exposure to BPA at submicromolar does not affect cellular viability. However, the genotoxic analysis showed that BPA induced an increase of DNA damage in HepG2 and MRC-5 cell lines [75]. Glucuronic acid conjugates and catechols are the major metabolites of BPs. Catechols can be further oxidized into BP-o-quinones, known genotoxic intermediates cable of binding to DNA nucleosides [76]. As the predominate oxidative metabolites of BPA, namely bisphenol A 3,4-quinone (also known as bisphenol-o-quinone, BPAQ), has been reported to reacts with deoxyguanosine via 1,4-conugate addition, forming adducts with DNA *in vivo* (Fig. 5). Atkinson and Roy [77] reported that *in vitro* incubation of DNA with BPA in the presence of peroxidase activation system can generated several adducts. They found that bisphenol A is oxidized by 70% to BPAQ, *in vitro* study. Chemically reacting BPAQ with deoxyguanosine monophosphate (dGMP) or DNA produced 6–8 BPAQ-DNA adducts, although their structures were not identified and characterized. Furthermore, as was shown by ^{32}P-postlabeling, BPA reacts with DNA to give adducts in CD1 male rats. Two major adducts from the liver matched those produced by the reacting bisphenol-o-quinone with dGMP or DNA.

BPAQ-DNA adducts have been detected *in vivo* by either BPAQ or metabolically activated BPA exposure using comet assay and ^{32}P Postlabeling technique. Mokra et al. [78] investigated DNA-damaging potentials of BPA and its analogues, including bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF) in human peripheral blood mononuclear cells (PBMCs) by using the alkaline and neutral versions of the comet assay. They found that BPs can cause DNA damage in human leucocytes by generating single strand-breaks (SSBs) and double strand-breaks (DSBs). BPA and BPAF at level as low as of 0.01 μg/ml are able to induce single strand-breaks [78]. Administration of BPA with a dose of 200 mg/kg body weight to mice for 8 days revealed that DNA adducts are formed in liver, whose content is about 3.4-fold higher than that of the control. DNA lesions formed in target mammary cells may bear relevance for the potential involvement of BPA in breast carcinogenesis [79]. Exposure with 200 μM of BPA, significantly (p value < 0.05) induces caspase-3-mediated cell death by inducing cytotoxicity, ROS, and DNA fragmentation. Higher levels of γ-H2AX and DNA tail damage indicated BPA's DNA damaging potential through an ATM/ATR/Chk1/*p*53-dependent pathway in BEAS-2B cells [80].

LC-MS based approaches were extensively developed for better understanding the chemical formation, transformation and biochemical behaviors of BPs–DNA adducts. Gross et al. identified these BPAQ-DNA adducts by using LC-Q-TOF and LC-IT mass spectrometry [81]. In mixtures of deoxynuclesides and deoxynucletides treated with BPAQ, reactions occur more readily with dGMP/dG followed by dAMP/dA. In the incubated mixture of ctDNA and BPAQ, BPAQ-guanosine adducts were distinctly detected, and apurinic sites (AP-sites) lesion was observed. Interestingly, BPAQ was found not to react with 2′-deoxycytidine (dC) and 2′-deoxythymidine (dT). Cai et al. [82,83] revealed BPA quinone metabolites can react with MCF-7 cell and form DNA adducts *in vitro*. The adducts of BPAQ with 2′-deoxyguanosine (dG), ct-DNA and genomic DNA of MCF-7 cell were identified and quantitatively detected by using EI-Orbitrap high-resolution MS and tandem MS/MS. The major DNA damages by BPA/BPAQ were identified as 3-hydroxy-bisphenol A-*N*7-guanine (3-OH-BPA-*N*7Gua) adducts. Based on the acquired mass data, MS/MS fragmentation pathway of 3-OH-BPA-N7Gua was elaborated. The findings provide evidences that BPA might covalently bind to DNA in MCF-7 cells via forming quinone metabolites, which may increase the understanding of health risk associated with BPs exposure [83].

Recent studies have also identified specific gene targets and found that they are epigenetically altered in response to BPA, which may increase breast epithelial proliferation and tumor development [84,85]. Exposure of primary human breast epithelial cells to BPA increased DNA methylation levels in the promoter region of the gene encoding lysosomal-associated membrane protein 3, LAMP3, and associated decreases in mRNA expression were detected [84].

3.3. DNA adductomics (unknown and unanticipated adducts)

Screening of unknown or unanticipated adducts in DNA samples led to the development of DNA adductomics. DNA adductomics is a relatively new field, involves the identification and quantitation of chemical modifications of DNA and the study of the factors from which they originate [10,11,16].

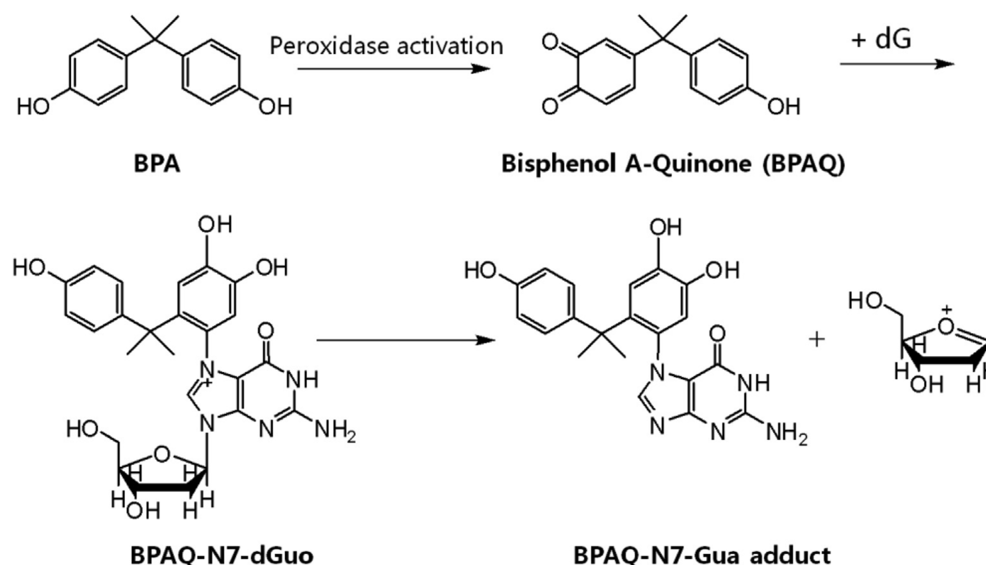

Fig. 5. Formation of bisphenol A-3,4-quinone (BPAQ)-dG adduct.

DNA adducts resulting from chemical exposure have been studied by untargeted MS, in which the detection of modified nucleosides in hydrolyzed DNA samples can be accomplished by neutral loss monitoring for the loss of 2′-deoxyribose (dR), the diagnostic structural feature of DNA adducts. LC-MS^n DNA adductomic studies are typically performed using triple quadrupole instrumentation operated in constant neutral loss (CNL) or "pseudo" CNL mode. Cooke et al. [86] proposed LC-QqQ-MS/MS strategy operating in constant neutral loss scan mode to simultaneously assess 2′-deoxyribonucleoside and nucleobase adducts in human urine. This method was also successfully applied to *in vitro* and *in vivo* studies to search for DNA adducts induced by methyl methanesulfonate (MMS) and five *N*-nitrosamines [86,87].

Turesky et al. [73,88] developed an untargeted, unbiased data-independent screening method, termed wide-SIM/MS/MS, which can screen for a wide-range of DNA adducts in human tissues. They have characterized and quantified DNA adducts of 4-aminobiphenyl (4-ABP), a bladder carcinogen, in DNA from fresh-frozen (FR) and formalin fixed paraffin embedded (FFPE) bladder tissue of bladder cancer patients and employed wide-SIM/MS/MS to screen for other DNA adducts that may form with 4-ABP and other AAs, including 2-naphthylamine (2-NA), 2-methylaniline (2-MA), 2,6-dimethylaniline (2,6-DMA), and several prominent HAAs, including PhIP, MeIQx, and AαC. This untargeted wide-SIM/MS/MS demonstrated the potent capability to identify DNA adducts induced by chemical exposure, which may contribute to the etiology of bladder cancer [73,88]. Wide-SIM scan events are followed by MS/MS scans to screen for modified nucleosides by co-eluting peaks containing precursor and fragment ions differing by −116.0473 Da, attributed to the neutral loss of deoxyribose. Wide-SIM/MS/MS was seemed to be superior in sensitivity, specificity, and breadth of adduct coverage to conventional methods with the LOD as low as 4 per 10^9 nucleotides.

An alternative is the use of high-resolution/accurate-mass (HRAM) MS^n for data acquisition, such as an Orbitrap mass analyzer, as demonstrated recently with a DNA adductomic approach using accurate mass monitoring of the neutral loss of the 2′-deoxyribose moiety (116.0474 amu) and subsequent MS^3 data acquisition. This approach provides a high level of selectivity allowing for minimal false positive detections, as well as accurate mass measurements for the determination of likely elemental compositions and mass spectral data useful for the elucidation of adduct structure. This new approach represents a dramatic improvement in terms of selectivity and identification capability and is an important advancement in the ability to investigate DNA damage in complex biological samples [89]. Li et al. [90] reported a comprehensive quantitation of pyridyloxobutyl-DNA phosphate adducts in five tissues from rats chronically treated with either (*S*)- or (*R*)-N′-nitrosonornicotine. Using a highly sensitive and specific LC−NSI−HRMS/MS method, they identified up to the maximum 32 unique DNA phosphate adducts which could be formed by 2′-hydroxylation of the N′-nitrosonornicotine enantiomers in each of 5 tissues from rats treated with these carcinogens for 10, 30, 50, or 70 weeks, with levels ranging from 780 ± 194 to 1010 ± 700 fmol/mg DNA. These data lead to a better understanding of the complex pattern of DNA damage induced by NNN during chronic treatment leading to tumor formation in rats. These new DNA adductomics approaches are expected to be useful for screening for DNA adducts induced by endogenous or exogenous exposures.

4. LC-MS-based sequencing of nuclobase modifications

Mass spectrometry-based methods demonstrated potentials in mapping nucleobase damages along DNA sequences, and are capable of providing additional structural information, facilitating mechanistic studies of the origins of DNA sequence specificity. A distinctive approach is MS-based analysis of exonuclease ladders of DNA chains, which relies on that DNA phosphodiesterases (PDEs) sequentially remove mononucleotides in either the 5′-to- 3′(e.g. bovinespleen phosphodiesterase) or the 3′-to- 5′(e.g. snake venom phosphodiesterase) direction. At a specific time point of exonuclease digestion, the digested products contain a mixture of DNA fragments differing from each other by one or more mononucleotides, forming DNA "ladders" in mass spectra. The mass differences between two adjacent peaks in this mass spectrum correspond to individual nucleotides, thus making it possible to sequence the whole oligos [91,92].

Despite MS sequencing experiments has achieved good results using the solution enzymatic digestion protocol, actually, the speed of solution enzyme digestion is not easy to control, and long time enzymatic digestion is prone to inactivation of enzymes. To overcome this problem, we fabricated a snake venom phosphodiesterase (SVP)-immobilized capillary bioreactor, which can be used for controlled cleaving oligodeoxynucleotides, for MALDI-TOF MS-based sequencing and for identification of damage sites in oligodeoxynucleotides [93]. By the application of capillary bioreactor of immobilized SVP, the sequence-specific modification of single-stranded oligodeoxynucleotide induced by a ubiquitous pollutant acrolein (Acr) was identified. Acr−33-mer adducts obtained from directly reacting 33-mer oligo with acrolein (1:20, in molar ratio) was subject to enzymatic digestion by the SVP bioreactor, and the formed fragments were further determined by MALDI-TOF MS analysis. Interestingly, only Acr−dG and Acr−dC adducts were observed in the adducted 33-mer oligo. The Acr adducts of dG and dC were distinguished from their specific mass shifts of $\Delta m/z = 426.16$ (theoretic mass of dG + Acr + K+, 425.20) and $\Delta m/z = 344.79$ (theoretic mass of dC + Acr, 345.20) in ladder. Thus, the sequence of the Acr adducted 33-mer oligo was identified as GCCCTGATTTT(C_{12}+Acr) AT(G_{15} + Acr + K)GG(G+K) CTAAGGGTCGCGGGA) (Fig. 6). This work demonstrates MS-based mapping method is promising applications in identification of sequence-specific damage, which may further our understanding of DNA damage caused mutagenesis.

5. Perspectives

The environmentally-induced genomic and epigenetic changes become increasingly relevant to human health and disease. LC-MS has played an important role in DNA modifications analysis. It has also become one of the preferred methods for qualitative and quantitative analysis of DNA adducts *in vivo* and *in vitro*. Mass spectrometry provides structural information about DNA nucleosides, nucleotides and bases, and modifications, thus contributing to identify and search for new DNA damages. In addition, MS-based DNA analysis, typically the LC-MS/MS coupled with the stable isotope dilution method, has evolved to be the *gold standard* for unequivocal identification and accurate quantification of DNA damages in biological samples. Benefited from LC-MS analysis, a series of land-marking discoveries have been made by identification and highly-sensitive quantification of DNA modifications induced by environmental exposure.

It is expected that in the near future mass spectrometry will further promote the development of DNA damage analysis in the following aspects: 1) Continuously improving the sensitivity and selectivity of mass spectrometry analysis, either from the point of view of chromatography or mass spectrometry, including adding mobile phase additives to improve separation and response, adjusting stationary phase to enhance selectivity and sensitivity, repressing chemical background, inhibiting metal ion adducts, and

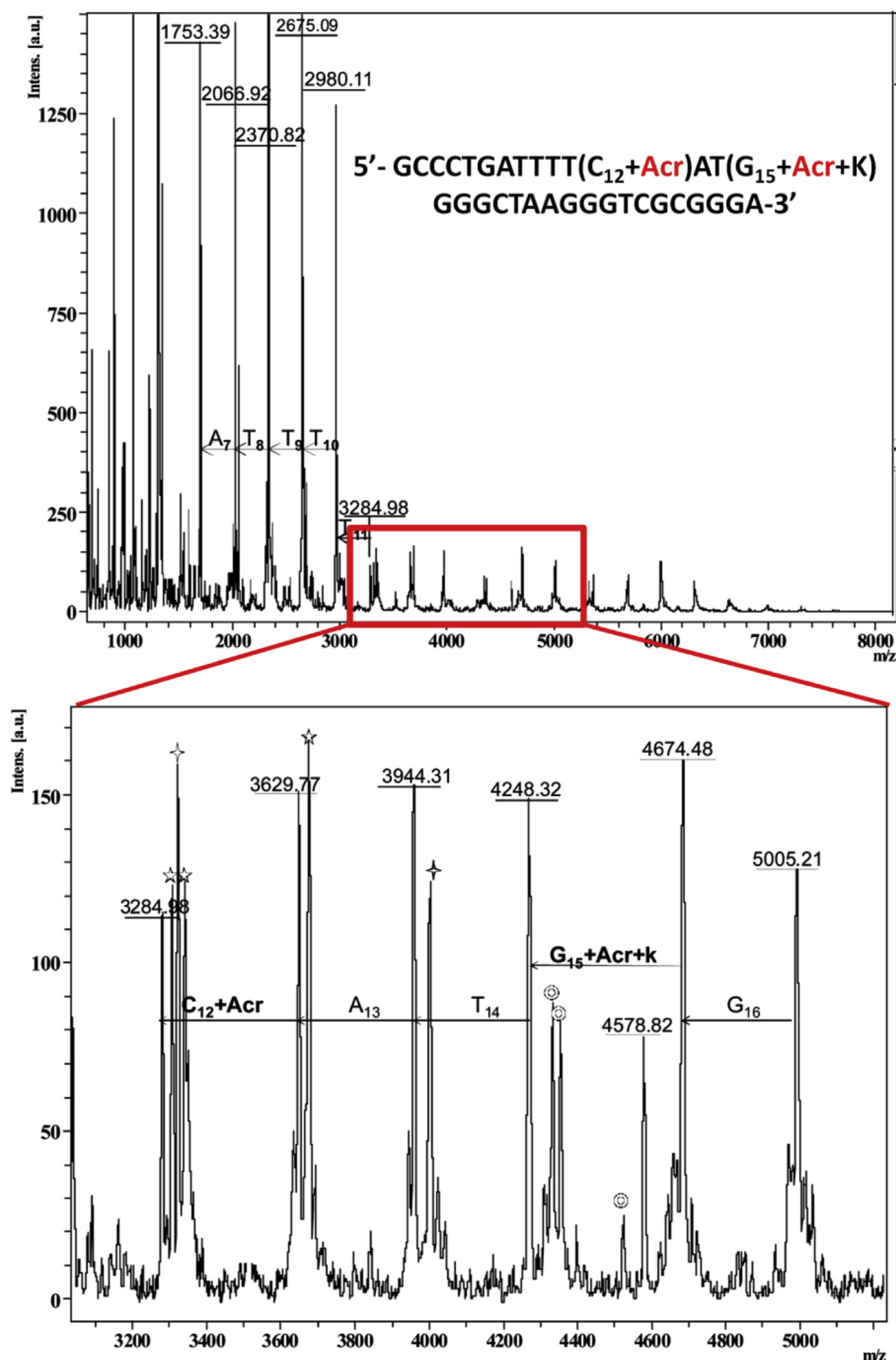

Fig. 6. Sequencing of Acr−33-mer adducts by SVP capillary bioreactors coupled with MALDI-TOF MS analysis, showing the identification of base-specific and sequence-specific modification induced by Acr. The subscripts of nucleotides (e.g., C12 and G15) were counted from the 5′-end. The cross stars, five-pointed stars, and concentric circles indicate K^+−oligodeoxynucleotide adducts, Na^+−oligodeoxynucleotide adducts, and unknown peaks, respectively. Reprinted with permission from ref. [93]. Copyright 2010 American Chemical Society.

enhancing MS signal response; 2) Developing potent MS methodology for the systematic analysis of DNA adductomics, since a large of environmental toxicants can induce a series of DNA modifications rather than limited types of damages; 3) Developing the integrated method involving online DNA digestion coupled LC-MS analysis, MS and MALDI-MS (3D) imaging; 4) Putting efforts in exploring genotoxicity of new emerging contaminants (e.g. nanomaterials and fine particles PM2.5), since their knowledge is in the ascendant and greatly complicated by the fact that it is a heterogeneous and often poorly described pollutant.

Overall, understanding the mechanisms of damage identification and repair is important to gain insight into the specific roles of DNA adducts in the development of cancer and other chronic diseases, because ultimately, the cellular repair capacity in response to exposure is critically related to the levels of DNA adducts in the genome or mutations in genes.

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