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The analysis of DNA adducts: The transition from ^{32}P -postlabeling to mass spectrometry

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Abstract

The technique of ^{32}P -postlabeling, which was introduced in 1982 for the analysis of DNA adducts, has long been the method of choice for in vivo studies because of its high sensitivity as it requires only $<10\text{ }\mu\text{g}$ DNA to achieve the detection of 1 adduct in 10^{10} normal bases. ^{32}P -postlabeling has therefore been utilized in numerous human and animal studies of DNA adduct formation. Like all techniques ^{32}P -postlabeling does have several disadvantages including the use of radioactive phosphorus, lack of internal standards, and perhaps most significantly does not provide any structural information for positive identification of unknown adducts, a shortcoming that could significantly hamper progress in the field. Structural methods have since been developed to allow for positive identification of DNA adducts, but to this day, the same level of sensitivity and low sample requirements provided by ^{32}P -postlabeling have not been matched. In this mini review we will discuss the ^{32}P -postlabeling method and chronicle the transition to mass spectrometry via the hyphenation of gas chromatography, capillary electrophoresis, and ultimately liquid chromatography which, some 30 years later, is only just starting to approach the sensitivity and low sample requirements of ^{32}P -postlabeling.

This paper focuses on the detection of bulky carcinogen-DNA adducts, with no mention of oxidative damage or small alkylating agents. This is because the ^{32}P -postlabeling assay is most compatible with bulky DNA adducts. This will also allow a more comprehensive focus on a subject that has been our particular interest since 1990.

Keywords

DNA adduct; ^{32}P -postlabeling; Gas chromatography; Capillary electrophoresis; Liquid chromatography; Mass spectrometry

1. Introduction

Exposure to carcinogens can lead to the formation of DNA adducts, a key step towards the onset of disease such as cancer. This is a well-established mechanism and therefore detection and monitoring DNA adducts can serve as an indicator of exposure and disease. While there is great debate as to whether the presence of DNA adducts will lead to disease,

it is imperative that early detection methods be developed to improve prognosis and early treatment. DNA adducts are an ideal target for human screening, biomarker discovery, and measurement of exposure, as adducts are often present well before the onset of disease.

1.1. Bulky aromatic DNA adducts

The term “bulky” DNA adducts was derived from the early experiments using ^{32}P -postlabeling that were aimed at the detection of adducts of polycyclic aromatic hydrocarbons (PAHs) whose identity could not be verified with this technique [1]. The term has been extended to include all relatively large aromatic and nonpolar carcinogen adducts. PAHs are a class of compounds produced from the incomplete combustion of organic material. They are found in a wide range of media ranging from cigarette smoke, environmental aerosols, and places of work [2–6]. The most studied PAH is benzo[a]pyrene (BaP), a well-known human and animal carcinogen (see structure, Fig. 1) [7,8]. BaP requires metabolic activation by CYP450s to its diol epoxide derivative before reacting with DNA [5,9].

Heterocyclic aromatic amines (HAAs) are formed by cooking protein-containing foods such as meat [10]. In 1986, Felton et al. [11] determined 2-amino-1-methyl-6-phenylimidazo[4,5- b]pyridine (PhIP) to be the major HAA in fried beef, at a level of 15 mg/kg (see structure, Fig. 1). PhIP is carcinogenic in mammalian models and requires metabolic activation before it can react with DNA [12,13]. 2-amino-3,8-dimethylimidazo [4,5- f]quinoxaline (MeIQx) is another well-studied carcinogen in this class and when fed to mice was shown to induce liver tumors (see structure, Fig. 1) [14]. The aromatic amine 4-aminobiphenyl (4-ABP) is a known human bladder carcinogen found in nanogram quantities in cigarettes (see structure, Fig. 1) [15,16]. 4-ABP reacts primarily with the C-8 position of guanine residues in DNA to form dG-C8-4-ABP.

DNA adducts are most often detected at the nucleoside or nucleotide (monomer) level. This provides optimal sensitivity and straightforward quantitation. However, DNA adduction is not random, and is highly sequence dependent. Sequence information is lost upon digestion to nucleosides or nucleotides and therefore analysis of oligonucleotides long enough for gene identification is also an important goal. While the bulk of this review is focused on the analysis of nucleotides and nucleosides, a discussion on the current progress toward the analysis of oligonucleotide adducts has been included.

2. Methods

The review by Apruzzese and Vouros [17] includes a very clear description of the need for a sensitive analytical method for the detection and quantitation of DNA adducts. As stated in that review, if an analytical method has a mass detection limit of 10 pg for a particular adduct, and a detection limit of 1 adduct per 10^8 nucleotides is required, this may require 10 g of tissue (1 g of tissue contains roughly 1 mg of DNA) [17]. For in vivo studies, and particularly human studies in which tissue samples are limited, the analytical method of choice must be superbly sensitive.

DNA adducts are formed at very low levels in living systems. Additionally, the DNA adducts are located amongst unadducted nucleotides generally at a minimal level of 10^6 times greater abundance. Therefore, an analytical method aimed at detecting DNA adducts in living systems must be very sensitive, highly specific, and require only small amounts of sample. This poses a daunting analytical challenge, and over the years several successful platforms have been developed. These include ^{32}P -postlabeling, gas chromatography mass spectrometry (GC-MS), capillary electrophoresis mass spectrometry (CE-MS), and liquid chromatography mass spectrometry (LC-MS). Each method has its own inherent strengths and weaknesses as we summarize here with a focus on sensitivity and sample requirements, two parameters important for application of the method to human studies, in which adducts are present at low quantities, and sample sizes are often limited. Table 1 summarizes the methods described in this review highlighting sample requirements and sensitivities of each method for different DNA adducts. Fig. 2 graphically represents these qualities.

2.1. The ^{32}P -postlabeling assay

In 1982, Gupta et al. [18] introduced the ^{32}P -postlabeling assay for the detection and quantitation of aromatic DNA adducts. Briefly, this assay works by digesting isolated DNA into 3'-deoxynucleoside monophosphates, transfer of γ - ^{32}P from adenosine [γ - ^{32}P]triphosphate to generate 3',5'-[5'- ^{32}P]diphosphates using T4 poly-nucleotide kinase [18]. The labeled adducted nucleotides are then separated by thin layer chromatography (TLC) and quantified by scintillation counting. This platform was capable of detecting one adduct in 10^7 – 10^8 nucleotides using only 1 μg of DNA [18].

A key limitation to the ^{32}P -postlabeling assay is the fact that all 3'-nucleoside monophosphates, both DNA and RNA, are substrates for T4 polynucleotide kinase and therefore the assay is dependent upon the ability to remove unadducted nucleotides from the reaction mixture. Successful improvements to this assay were soon developed by including additional cleanup steps before the labeling reaction, which reduced the presence of unmodified nucleotides in the sample.

In 1985, Gupta [19] improved the sensitivity of the ^{32}P -postlabeling assay by extracting the adducted nucleotides with 1-butanol in the presence of tetrabutyl-ammonium chloride before the labeling reaction. This extra step extended the detection limit to a single adduct in 10^9 – 10^{10} nucleotides while maintaining the extremely low sample requirements of only 1–10 μg of DNA [19]. In 1986, Reddy and Randerath [20] used Nuclease P1 enrichment to achieve similar sensitivity and in 1988 [21], reversed-phase high performance liquid chromatography (HPLC) was used for the separation of unadducted from adducted nucleotides, as well as for separation of adducts with different structures into individual fractions which were then visualized on TLC plates, significantly diminishing the detection complexity. Fig. 3 shows data comparing butanol and nuclease P1 enrichment in conjunction with ^{32}P -postlabeling in a study of DNA adducts from tobacco chewers [1,21]. In this case, butanol enrichment was proven to be superior to nuclease P1 enrichment in the isolation of adducts from oral mucosa [1,21]. While nuclease P1 treatment works well with PAH adducts, it does not work well with aromatic amines or C8-dG adducts [22–24].

³²P-Postlabeling has since been the method of choice, particularly with in vivo studies, due to its high sensitivity (1 adduct in 10¹⁰ nucleotides) and low sample requirement (1–10 µg DNA) and has been used in a number of important studies for biomonitoring, measures of exposure, and linking DNA adduction to disease as illustrated in the reviews by Beach and Gupta [1] and Gyorffy et al. [25]. However, ³²P-postlabeling, like any analytical method, does have some disadvantages. The assay requires the use of ³²P, a radioactive isotope that poses health risks and requires a dedicated, isolated environment [26]. While intrinsically quantitative, internal standards are not available which makes it difficult to account for losses during sample preparation or incomplete labeling, which is likely responsible for the underrepresentation of DNA adducts that have been observed when comparing this assay to other methods [27–29]. Perhaps the most significant disadvantage to this assay is the lack of structural information, which, before structure-based methods were available, often led to ambiguity in the identities of the DNA adducts that were being detected.

2.2. Gas chromatography mass spectrometry

Gas chromatography has been a powerful separation technique since its introduction in the 1950s and its true power was realized when it was hyphenated with electron capture ionization and mass spectrometry, the most common mass analyzers being ion traps or single quadrupoles. GC–MS provides excellent resolution and peak capacity of volatile molecules as well as structural information, screening capabilities, and even exact mass when combined with a high resolution time of flight (TOF) analyzer. In the analysis of DNA adducts, GC–MS suffers from low throughput due to the requirement to derivatize these polar adducts combined with relatively long temperature gradients. In addition, GC is only compatible with volatile molecules, a stipulation that poses problems when trying to analyze polar nucleotides and nucleosides, and indirect strategies must be applied. Despite these limitations, GC–MS has achieved widespread use for the analysis of DNA adducts. In the analysis of PAH adducts, PAHs are liberated from the DNA by acid hydrolysis and then detected as derivatized tetrols. Not long after its introduction, this method was used to confirm the presence of BaP adducts in human placenta [30]. In this study the importance of the structural elucidation capability of GC–MS was clearly illustrated when HPLC–synchronous fluorescence spectroscopy was used to screen for BaP adducts and GC–MS was used to confirm their presence in 10 of 28 samples [30]. This study provided conclusive evidence that maternal exposure to environmental carcinogens can lead to DNA adducts in human placenta [30]. GC–MS was used to show that, after human exposure from tobacco smoke, BaP is transported to the cervix where it is metabolized to BPDE and forms DNA adducts [31]. The authors cite previous studies of DNA adducts in human cervical cells using ³²P-post-labeling [31–35]. However, since the analysis was conducted on the released BaP tetrols, the exact structural identity of the DNA adducts remained unknown. Still, GC–MS was able to confirm that adducts derived from BaP were likely at least one of the markers detected by the studies utilizing ³²P-postlabeling [31].

GC–MS has proven useful for bulky DNA adducts of different classes. Lin et al. [36] introduced a method for the detection of 4-ABP adducts in human urinary bladder and lung tissues. In this method, 0.05 N NaOH was used to hydrolyze 4-ABP from the DNA molecules [36]. Gas chromatography negative ion chemical ionization mass spectrometry

(GC–NICI–MS) was used to detect the liberated carcinogens with a sensitivity as low as 0.32 adducts per 10^8 nucleotides [36]. This method was validated by comparison with the long established ^{32}P -postlabeling assay and excellent agreement was found between the quantitation results from the two methods ($r = 0.999$, $n = 9$) [36].

The alkaline hydrolysis method and GC–NICI–MS was later used by Friesen et al. [37] to detect and quantify the heterocyclic aromatic amine PhIP in various human tissues. Fig. 4 shows the selected ion monitoring (SIM) chromatograms of derivatized PhIP and the internal standard isolated from the DNA of rat lung. Excellent sensitivity was reported for the GC–MS detection of this adduct as well, with an LOD of 1 adduct in 10^8 nucleotides when using 100 μg of DNA and sensitivities were comparable to that of ^{32}P -Postlabeling (1 adduct in 10^8 nucleotides) when 3 μg of DNA was used [37]. The same authors also found a good correlation when comparing the GC–MS method with ^{32}P -postlabeling ($r^2 = 0.83$) [37]. As the authors point out, best results were achieved when using at least 100 μg of DNA so this GC–MS method may not be practical for most human studies, in which smaller amounts of DNA can be obtained [37].

The primary limitations preventing GC–MS from becoming the method of choice for the analysis of DNA adducts in biological matrices are the indirect methods by which DNA adducts must be measured (e.g. hydrolysis to tetrols and removal of sugars) and the relatively large amounts of sample needed to reach the sensitivity required to measure adducts at biologically relevant levels [38].

2.3. Capillary electrophoresis mass spectrometry

In 1994, Wolf and Vouros [39] were among the first to use capillary zone electrophoresis (CZE) and continuous flow fast atom bombardment mass spectrometry (FAB-MS) for the detection of low picogram amounts of standard DNA adducts of three different classes (4-ABP, N-acetylaminofluorene (AAF), aminofluorene (AF), anthracene). While low mass sensitivity was achieved, the limitation of CE was realized as the concentration detection limit was on the order of 10^{-5} M due to the requirement of 5–10 nL injection volumes. The sample stacking technique (concentration of analyte into a plug) proposed by Chien and Burgi [40] was later used to partially overcome this problem and reach concentration detection limits on the order of 10^{-8} M for adducts of different classes, a nearly 1000 $\times$ improvement [41,42]. Fig. 5 shows the mass electropherogram of a digested and purified in vitro reaction of BPDE with calf thymus DNA. Multiple reaction products were detected in addition to the expected BaP-dGMP using sample stacking and this demonstrates the powerful resolving capabilities and ability to detect unknown or unexpected DNA adducts, as these results were obtained by scanning a wide mass range from 400 to 1400 m/z [42].

Among the electrophoretic methods, capillary electrochromatography (CEC) is a separation technique that essentially combines traditional HPLC with electrophoretic mobility resulting in less band broadening than that observed with HPLC and, before the advent of ultrahigh pressure liquid chromatography (UHPLC), allowed the use of sub 3 μm particles without the backpressure issues seen with HPLC separation [43]. In 1997, Ding and Vouros [44] explored the fundamentals of CEC for hyphenation with MS in the analysis of model carcinogens, AAF-dG and the PAH benzo[g]chrysene. CEC demonstrated several

advantages over HPLC including higher resolution (plate counts of >100,000), faster analysis (seconds vs. minutes) and lower analyte consumption [44]. Utilizing on-column focusing and step gradients the authors achieved concentration detection limits on the order of 10^{-6} M and shed light on key physical properties for the improvement of sensitivity in CE-MS [44,45].

In a more recent application, Fang et al. [46] used CE-MS to characterize an adduct resulting from the PAH metabolite 9-OH-BaP which had been observed previously in rodent liver and lung tissue by ^{32}P -postlabeling after exposure to BaP [47,48]. This study once again showed the importance of a structure-based method to confirm the identity of unknown adducts.

2.4. Liquid chromatography mass spectrometry

High sensitivity has been achieved in all the methods discussed to this point, but with each method, the researcher must make a sacrifice and either use large amounts of sample or lose the ability for structural identification. Clearly a more effective method would combine all three attributes: high sensitivity, low sample requirements, and structural identification. A number of publications have identified the increasing role that LC-MS would play in the analysis of DNA adducts and the evolution of the technique is summarized next [17,49–53].

2.4.1. Conventional flow liquid chromatography mass spectrometry—HPLC has been used for the analysis of DNA adducts since the early 1990s and hyphenation with MS began to show great promise for unambiguous identification of DNA adducts in complex matrices. The coupling of LC with ESI dramatically increased the utility of LC-MS for the detection and quantitation of DNA adducts. In 2001, Nelson et al. [54] used LC-MS/MS to confirm the bioactivation of N-OH-PhIP and formation of DNA adducts in human prostate and human prostate-derived cells and provide insights into protection against early-stage prostate carcinogenesis. The analytical method provided an LOD of 2 adducts in 10^7 nucleotides using 100 μg of DNA [54]. Paehler et al. [55] utilized LC-MS/MS using a 1 mm id $\times$ 50 mm reversed-phase column and a flow rate of 50 $\mu\text{L}/\text{min}$ to identify the major DNA adducts associated with MeIQx exposure, and were the first to provide spectral data on HAA-DNA adducts using 100 μg of DNA to achieve similar sensitivity [26]. In 2005, Beland et al. [56] used HPLC-ESI-MS/MS to detect BaP-dG adducts in lung DNA from mice fed a diet supplemented with coal tar and from human lung tissue samples from both healthy autopsy samples and lung cancer patients. Using 100 μg of DNA, this platform achieved an LOD of 0.3 adducts per 10^8 nucleotides which enabled the detection of dG-BaP adducts in 1 of the 26 human samples while ^{32}P -post-labeling revealed adducts in 20 of the 26 human samples [56]. These results suggest that BaP-related adducts constituted only a small fraction of the DNA adducts which points to the low specificity of ^{32}P -postlabeling compared to LC-MS/MS [56]. Singh et al. [29] developed a narrow bore LC-ESI-MS/MS method to detect N2-BaP adducts in mouse liver with a detection limit of 3 adducts in 10^8 nucleotides using 50–100 μg of DNA. A dose-dependent trend was identified and their results correlated well with their analysis of the same sample set using ^{32}P -postlabeling [29]. Singh et al. [57] later undertook a major study of the effects of environmental air pollution

on human populations from three cities in Eastern Europe. Interestingly, they found negative correlations between oxidative DNA damage and total PAH levels and BaP adducts [57].

Recent publications show that LC–MS with conventional flow using 1–4.6 mm columns is still a valuable platform for in vivo animal studies. In 2011, Zhang et al. [58] used a stable isotope dilution LC–MS/MS method to detect DNA adducts in oral tissue of mice resulting from exposure to dibenzo[a,l]pyrene (DB[a,l]P). They were able to determine the primary DNA adduct resulting from this exposure as anti-DB[a,l]PDE-N6-dA and quantify its abundance. While the authors do not explicitly state their LOD, the lowest point on their calibration curve was 5 pg in 100 µg DNA [58].

2.4.2. Capillary and nano-flow liquid chromatography mass spectrometry—In the mid 1990s researchers began to capitalize on the advantages offered by low-flow LC and ESI to improve sensitivity of DNA adduct detection, perhaps with inspiration from capillary electrophoresis techniques which had already demonstrated the compatibility of MS with these lower flow rates. Probably the earliest use of capillary LC (capLC) coupled with tandem MS and microESI (µESI) for the analysis of DNA adducts was done by Wolf and Vouros [39]. AAF-DNA adducts were separated by a capillary column with the dimensions of 320 µm i.d. × 15 cm packed with 3 µm C18 beads at a flow rate of 4 µL/min provided by a syringe pump [39]. Continuous flow fast atom bombardment (CFFAB) was used for ionization due to its optimal low flow of ~5 µL/min which was nicely compatible with MS. The authors also investigated the advantages of the different MS scan modes offered by a triple quadrupole mass spectrometer, namely constant neutral loss (CNL) and multiple reaction monitoring (MRM) [39]. Fig. 6 (top) shows the use of CNL for screening a mixture for the presence of AAF adducts while Fig. 6 (bottom) shows the dramatic sensitivity enhancement of MRM for the specific transitions ($473\text{ }m/z \rightarrow 357\text{ }m/z$) and ($489\text{ }m/z \rightarrow 373\text{ }m/z$) [39]. The method suffered from relatively poor sensitivity and large sample requirements, with an LOD in MRM mode of 1 adduct in 10^6 nucleotides using 1 mg of DNA. However, this study provided an excellent illustration of the applicability of capLC–µESI–MS/MS for the analysis of DNA adducts and the unique features offered by the triple quadrupole analyzer for the analysis of this class of compounds [39]. Rindgen et al. [59] detected dG-PhIP adducts formed in vitro using capillary LC–ESI–MS/MS with an LOD of 80 fmol using 1 mg of DNA (three adducts per 10^8 nucleosides). ^{32}P -postlabeling was performed in parallel and proved to be much more sensitive using only 3.75 µg of DNA [59]. Still, the LC–MS/MS method proved sufficiently sensitive for in vivo exposure studies in animals and provided important structural information that enabled the detection and quantitation of three distinct PhIP-DNA adducts [59]. Gangl et al. [60] helped to revitalize the interest in capLC by decreasing the flow rate to 0.250 µL/min for nanoESI (referred to in the publication as microelectrospray). The same authors also investigated the sensitivity associated with different tandem MS experiments in the triple quadrupole mass spectrometer. CNL was 1000× less sensitive than single reaction monitoring (SRM) for the analysis of dG-IQ, 1 adduct in 10^4 nucleotides vs. 1 adduct in 10^7 nucleotides, respectively, using 300 µg of DNA [60]. This was a very important step because, as also pointed out in other reviews, SRM in a triple quadrupole has become the most accepted MS/MS technique

for clinical applications and is regarded as the most sensitive and specific MS method in DNA adduct detection and quantitation [61].

In a further reduction in column diameter, Soglia et al. [62] used capLC–nano-ESI–MS/MS (referred to in the publication as μ ESI) to achieve an LOQ for dG-C8-IQ (2-amino-3-methylimidazo[4,5-f]quinoline) of 2 adducts in 10^8 nucleotides. HPLC separation was done on a 75 μ m i.d. self-packed reversed-phase column at a flow rate of 200 nL/min for introduction into the mass spectrometer [62]. This method was used to measure adduct formation in rat liver as a function of IQ dose and results were compared with a similar study using 32 P-postlabeling [62]. A relatively large quantity (300 μ g) of DNA was required to achieve this sensitivity [62]. Fig. 7 shows a comparison between 32 P-postlabeling and capLC–nanoESI–MS/MS of dG-IQ adducts from pancreatic tissue of non-human primates [26]. In the study by Gangl et al. [53], the strengths and weaknesses of each method are clear. Both methods had comparable detection limits, roughly 1 adduct in 10^9 nucleotides (sides/tides) which demonstrated the applicability of LC–MS to in vivo human and animal studies just as 32 P-postlabeling had been used for decades [53]. However, to achieve this level of sensitivity, the LC–MS platform required 500 μ g of DNA compared to the 30 μ g used for the 32 P-postlabeling assay [53]. The improved TLC separation developed by Turesky et al. [63] was capable of resolving the N2 from C8 isomers, which was readily accomplished with LC [53]. However, the LC–MS assay provided better resolution of the adducts (reversed-phase column compared to TLC plate) as well as molecular weight information allowing for the unambiguous identification of the adducts [53]. Wang et al. [64] used nanoflow LC–ESI with a quadrupole time of flight mass spectrometer (QTOF) for the detection and quantitation of BaP-dN adducts in mouse lung tissue after exposure to asphalt fume. The authors achieved femtomole-level detection limits in part by utilizing a 75 μ m id capillary column however a large amount of DNA (1 mg) was still utilized [64]. Perhaps the large sample was required due to the extensive offline cleanup steps aimed at enriching multiple BaP-dN adducts [64]. Ricicki et al. [65] used capLC– μ ESI–MS/MS to detect and quantify dG-C8-ABP adducts in human pancreatic tissue and achieved a detection limit of 5 fmol dG-C8-4-ABP (5 adducts per 10^9 nucleosides) with a 320 μ m id capillary column and a flow rate of 10 μ L/min. This was one of the first studies using LC–MS to study 4-ABP adducts in human samples and demonstrated the viability of this platform for screening human samples. However, in this study sample quantities were not limited and 300 μ g of DNA was procured from donated organs for digestion and analysis [65]. For human screening this will rarely be the case as non-invasive techniques are preferred.

2.4.3. Column switching—One of the drawbacks in many of the studies utilizing LC–MS was the fairly large sample requirements, often 100 μ g of DNA or more. Sample requirements of this size are simply incompatible with most human studies and so these requirements would have to be significantly decreased to gain acceptance and compete with 32 P-postlabeling. One factor dictating the amount of sample that is needed for an analytical method is the cleanup requirement which may involve one or more offline steps that can include solid phase extraction (SPE), liquid–liquid extraction, and protein precipitation [36,39,59,66–68]. Not only does this offline cleanup result in analyte losses, but it also significantly increases analysis time, reducing throughput. In 1997, Vanhoutte et

al. [50] showed that a nanoflow ESI–LC–MS system with online column switching could improve the mass detection limit of BPA–dG by 3300×, as compared to conventional ESI (200 nL/min vs. 40 µL/min, respectively). Doerge et al. [69] used narrow bore (2 × 150 mm column) LC–ESI–MS and online column switching to detect 4-ABP adducts in hepatic DNA from mice treated with 4-ABP. A detection limit of 0.7 adducts in 10⁷ nucleotides using 100 µg of DNA and adduction levels ranging from 4.9 and 30 dG–C8–4-ABP adducts in 10⁷ were detected in 0.1 mg 4-ABP/mouse and 1.0 mg 4-ABP/mouse, respectively [69]. The authors demonstrated the exceptional cleanup provided by online column switching and faster overall analysis time by directly injecting the enzymatic DNA hydrolysates without protein precipitation or any offline cleanup [69]. Additionally, as the authors mentioned, improved selectivity in cleanup can be accomplished by using different online cleanup strategies, such as immunoaffinity chromatography [69].

After the initial introduction of online column switching, significant effort was placed into decreasing sample requirements which, in effect, has made LC–MS much more compatible with human studies. In 2010, Singh et al. [70] use narrow bore LC–ESI–MS/MS and online column switching/cleanup for the analysis of dG–C8–PhIP to achieve a detection limit of 1.5 adducts in 10⁸ nucleosides using 50 µg of DNA. This method was used to quantify dG–C8–PhIP in colon tissue of mice treated by oral gavage with PhIP for 5 days [70]. Adduction levels of roughly 15 adducts in 10⁶ were found in these tissues using a very rapid and easily automatable method [70]. Randall et al. [68] utilized an Agilent Technologies microfluidics HPLC chip, which incorporates online SPE, capLC and nanoESI, all with minimal dead volume. With this technology, sample losses were minimized and sensitivity was maximized, along with improving the reproducibility and stability of nanoESI. The true value of this method is the ability to achieve a detection limit for dG–C8–4-ABP of 5 adducts in 10⁹ nucleosides using only 5 µg of DNA (1.25 µg of DNA per injection) [68]. With online sample cleanup and nanoESI, the authors developed an LC–MS platform with sensitivity and low sample requirements competitive with ³²P-post-labeling making this applicable to human studies, but with the added benefit of positive adduct identification through structural information (MW, fragmentation). This platform was later used to study the chemopreventative effects of sulforaphane, on dG–C8–4-ABP formation in mouse bladder cells, and later to identify the role of Nrf-2 directed cytoprotection against 4-ABP in different mouse tissues [71].

2.4.4. Ultra performance liquid chromatography mass spectrometry—A

fledgling technique compared to most of those discussed in this review is UPLC. This technique introduced in 2004 has been quickly gaining popularity due to the fast analysis times (<10 min) and high-resolution separation efficiencies (>100,000 plates) compared to traditional HPLC. This is afforded by using sub 3 µm packing material in the analytical columns and specially designed instrumentation to withstand the high pressure (<15,000 psi). In 2011, Nauwelaers et al. [72] used µUPLC–µESI–LC–MS/MS to study the formation of 4-ABP and several HAA adducts in rat and human hepatocytes. Using only 10 µg of DNA and offline SPE, an LOQ of 1 adduct in 10⁷ nucleosides (assumed from lowest point on calibration curve) the authors compared quantities of DNA adducts in human and rat tissue [72]. They found that rat models may significantly under-represent adduction levels in

humans, as HAA adducts were as much as 100-fold higher in human hepatocytes as compared to rat [72]. Yun et al. [73] used μ UPLC–MS/MS and online sample enrichment to develop a highly sensitive method with an LOD of 0.3–1 adducts per 10^8 nucleosides using only 10 μ g of DNA. The authors used this platform to quantify aristolactam DNA adducts in the tissues of mice exposed to 8-Methoxy-6-nitrophenanthro-[3,4-d]-1,3-dioxolo-5-carboxylic acid (AA-I), a carcinogen associated with rare cancers of the upper urinary tract [73]. More significantly, they demonstrated the utility of this method for human biomonitoring by analyzing DNA taken from the renal cortex of patients from Taiwan, the country where the largest number of these cancer cases are reported [74]. Levels of dA-AL-I were found ranging from 9 to 338 adducts per 10^8 nucleosides, while dG adducts were not found [73]. Herrmann et al. [75] used isotope dilution UPLC–MS/MS to investigate the activation of the secondary herbal metabolite methyleugenol by sulfotransferases and subsequent formation of DNA adducts. Using a 2.1×100 mm Phenyl column packed with 1.7 μ m particles and a flow rate of 300 μ L/min, an LOD of 2–6 adducts in 10^8 nucleosides was achieved starting with only 12.5 μ g of DNA [75].

While only a few papers have been published at the time of this review utilizing UPLC–MS for the detection of DNA adducts, this is certain to be a powerful technique which will help bridge the gap in sensitivity, speed of analysis, and low sample requirements between ^{32}P -postlabeling and MS-hyphenated techniques.

3. Mass spectrometry in the analysis of oligonucleotide adducts

The analytical approaches discussed to this point, both ^{32}P -postlabelling and MS methods, although very sensitive and selective, are limited to the determination of DNA damage by the analysis of DNA adducts of monomeric species. In addition to quantitative data, MS-based methods have been successful in providing structural information about the adducts, i.e. linkage to sugar, phosphate or nucleobase. While this information is proving to be of great value in terms of risk assessment, it should not be over-looked that a significant contribution of the mutagenic effect of a carcinogenic agent is also dependent on the sequence selectivity in the adduction process and the location of the carcinogen within a DNA sequence. Thus, a successful technique for predicting the relationship between DNA adduction and cancer is one which would be sensitive enough to quantify the exposure limit, explore whether the covalent interaction is modifying the nucleotide, probe the efficiency of the cellular repair process for removing the DNA adduct and last, but not necessarily least, determine the exact location of adduct attachment on the DNA. It is only by addressing these issues simultaneously that the DNA adduction can be understood and new tools for the early detection of cancer can be found. In this regard the detection and structural characterization of oligonucleotide adducts plays a major role.

Over the last few years there has been rapid progress in the development of sequencing techniques but unfortunately, in these next-generation DNA sequencing or PCR-based techniques, the adduct is lost prior to DNA sequencing [76,77]. It is this inherent failure of these techniques to analyze DNA adducts which has brought MS to the forefront as an efficient technique to sequence DNA adduct fragments. Fragmentation patterns are well defined and nomenclature based on the dissociation of unmodified oligonucleotides

introduced by McLuckey can be incorporated for the interpretation of CID spectra of adducted oligonucleotides [78].

For more than a decade now, the Vouros research group has been continuously working to advance the role of mass spectrometry in analyzing oligonucleotide adducts. In 1998, Marzilli et al. [79] analyzed the unmodified oligonucleotide 5'-CCGGAGGCC and the aflatoxin B1 (AFB) modified oligonucleotides using a Finnigan LCQ ion trap mass spectrometer. Later in 2000, Harsch et al. reported the use of LC-MS/MS for the identification of benzo[c]-phenanthrene diol epoxide adducted double-stranded (ds) 12-mer oligonucleotide (TAGTCAAGGGCA, sequence derived from the HPRT gene sequence) using a reversed-phase polystyrene divinylbenzene (PS-DVB) particle column (1.0 × 150 mm, 3 µm) and 20 mM ammonium acetate as an ion pairing reagent [80]. Further in 2007, Xiong et al. [81], reported separation and sequencing of (±)-anti-7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene ((±)-anti-BPDE) adducted ds 5'-PO4—ACCCGCGTC¹⁵⁷CGCGC-3' oligonucleotides containing codon 157 of the p53 gene using PS-DVB monolithic columns by ion-pair reversed-phase nanoHPLC coupled to ion trap mass spectrometry. Recently Sharma et al. [82] evaluated a number of ion pairing agents for the separation and sequencing of AAF modified isomeric oligonucleotide adducts. The latter study also discusses optimization of mobile phase conditions for the positional mapping of oligonucleotide adducts individually or in a complex mixture using a PS-DVB monolithic column for reversed-phase ion-pair liquid chromatography electrospray ionization tandem mass spectrometry [82]. In addition, this study also reported using the optimized mobile phase conditions for the positional mapping of oligonucleotide adducts individually or in a complex mixture. Linscheid et al. reported a number of studies starting in 1995 regarding the use of CZE and negative ion ESI-MS for detecting styrene oxide adducted dinucleotides–heptanucleotides [83,84].

In 2007, Chowdhury and Guengerich [85] reported direct detection and mapping of sites of base modification of a (±)-anti-BPDE or 4-ABP adducted double stranded oligonucleotide containing codon 157 of the p53 gene using an Acquity UPLC BEH C18 column (1.7 µm, 1.0 mm × 100 mm) and 10 mM ammonium acetate as the ion pairing reagent. In 2008, Jamin et al. [86] investigated the influence of the local environment and neighboring base effect on the adduction of HAAs, specifically PhIP and IQ on T-rich model single-strand oligonucleotides. Similarly, Chan et al. [87] reported an electrospray ionization tandem mass spectrometry method for the identification and position mapping of unmodified and aristolochic acid modified (5'-TTTATT-3', 5'-TTTGTT-3' and 5'-TACATGTGT-3') oligonucleotides. Anichina et al. [88] used a 4000 QTRAP triple-quadrupole mass spectrometer and a triple TOF 5600 hybrid quadrupole time-of-flight (TOF) mass spectrometer for the analysis of the binding interaction of oligonucleotides with structurally related bromobenzoquinones. Lai et al. [89] reported a similar study on electrospray ionization tandem mass spectrometric characterization of DNA adducts formed by bromobenzoquinones in 2011.

Despite the progress that has been achieved toward LC-ESI-MS/MS analysis of oligonucleotide adduct mixtures manual mass spectral interpretation of the resulting data is a bottleneck limitation. Several groups including the Vouros research group have also been

working on the development of data mining software of mass spectrometric data for nucleic acids for the last few years [90–95]. For example GenoMass software, based on the ‘reversed pseudo-combinatorial’ approach developed in the Vouros lab is a fast, automated, efficient, high-throughput data-mining algorithm for mass spectrometric analysis of nucleic acids [94,95]. This peak assignment software can further be utilized for tandem mass spectrometry data computational interpretation of oligonucleotide adducts thus, not only identifying the oligonucleotide but also determining the exact location of the modification [82].

Mass spectrometry coupled to separation techniques like liquid chromatography along with efficient LC–MS/MS data mining software could result in a significant step forward in genomic DNA research. The MS coupling to capLC helps in minimizing ion suppression, which could result from co-eluting similar compounds, or isomeric oligonucleotide adducts. The ability to couple LC–MS/MS with efficient data interpretation software like Geno-Mass is critical to the future of the field because of the huge amount of data needed to be interpreted for the digested genomic DNA. It is only by addressing and solving the challenges associated with building a mass spectrometry-based platform for analyzing digested genomic DNA fragments that the interaction between genes and environment could be understood.

4. Conclusions and future directions

Since its introduction in 1982, ^{32}P -postlabeling has been the preferred method for the analysis of bulky DNA adducts, particularly in human studies due to its low sample requirement of $<10\text{ }\mu\text{g}$ and high sensitivity of 1 adduct in 10^{10} nucleotides. Ambiguity in the identification of DNA adducts has led to the hyphenation of GC, CE and LC with MS to provide molecular weight and structural information for DNA adducts in question. GC–MS is inherently not ideal for the analysis of polar DNA adducts which require derivatization. For adequate sensitivity GC–MS requires relatively large amounts of DNA, as much as 100–300 μg and, in addition, when it comes to bulky adducts, it is effectively an indirect method since the analysis is conducted on the PAH tetrol or other moiety produced by hydrolysis of the adduct. Various CE–MS techniques including CEC and CZE have helped reduce the amount of DNA required for analysis and provided highly efficient separation with plate counts $>100,000$. CE with its low flow rates is fully compatible with nanoESI–MS and provided increased sensitivity with smaller sample sizes. However, this technique often proved troublesome because of interferences from complex biological matrices. LC–ESI–MS is a highly flexible technique capable of analyzing a wide range of DNA adducts in a variety of different biological matrices. Conventional LC–MS using 1–4.6 mm columns and compatible flow rates still required 100–300 μg of DNA and LODs 100–10,000 $\times$ higher than ^{32}P -postlabeling. Capillary separation methods combined with micro or nanoESI have produced a significant improvement in sensitivity. UPLC–MS has reduced analysis times and improved separation efficiencies with plate counts of $>100,000$. Perhaps the most significant development which has helped close the gap between ^{32}P -postlabeling and LC–MS was the introduction of chip-based technologies for online column switching which combine online sample cleanup, capillary separation, and nanoESI. This has enabled detection limits of ~ 1 adduct in 10^9 nucleotides using 1–10 μg of DNA.

The continuous developments in mass spectrometers will continue to improve the sensitivity of existing analytical methods. It is clear that technological developments in LC–MS, perhaps combining UPLC with chip-based sample cleanup and capillary chromatography will continue to push the lower limits of detection of DNA adducts while reducing the amount of sample required for analysis making LC–MS the preferred technique for noninvasive human biomonitoring and screening using biological fluids such as urine. Perhaps LC–MS will also provide clinicians with a tool for the early detection of disease and dangerous exposures to environmental carcinogens and the ability to sequence DNA adduction sites may provide a better understanding of the relationship between DNA adduction and disease.

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Abbreviations

PAH	polycyclic aromatic hydrocarbon
BaP	benzo[a]pyrene
BPDE	benzo[a]pyrene diol epoxide
HAA	heterocyclic aromatic amine
PhiP	2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
4-ABP	4-aminobiphenyl
dG	deoxyguanosine
MS	mass spectrometry
CE	capillary electrophoresis
LC	liquid chromatography
TLC	thin layer chromatography
HPLC	high performance liquid chromatography
NICI	negative ion chemical ionization
TOF	time of flight
SIM	selected ion monitoring
CZE	capillary zone electrophoresis
AAF	N-acetylaminofluorene
AF	aminofluorene
dGMP	deoxyguanosine monophosphate
CEC	capillary electrochromatography

UHPLC	ultrahigh pressure liquid chromatography
LOD	limit of detection
ESI	electrospray ionization
MS/MS	tandem mass spectrometry
DB[a,l]P	dibenzo[a,l]-pyrene
dA	deoxyadenosine
CNL	constant neutral loss
MRM	multiple reaction monitoring
SRM	single reaction monitoring
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
QTOF	quadrupole time of flight
PS-DVB	polystyrene divinylbenzene
ds	double-stranded
dN	deoxynucleotide
SPE	solid phase extraction
UPLC	ultra performance liquid chromatography
AA-I	8-methoxy-6-nitrophenanthro-[3,4-d]-1,3-dioxolo-5-carboxylic acid
AL-I	aristolactam
QTRAP	quadrupole linear ion trap

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Fig. 1. Structures of several bulky DNA adducts discussed in this review. From top left to bottom right, the aromatic amine dG-C8-4-ABP, the PAH N2-BaP-dG and two heterocyclic aromatic amines, dG-C8-PhIP and dG-C8-MeIQx.

Fig. 2.

Plot of the DNA adducts and methods used to detect them. Points closest to the x,y origin represent the most sensitive methods with the smallest sample requirements. The number of DNA adducts per nucleo(side/tide) is plotted (x -axis) vs. the amount of DNA in μg (y -axis). CS stands for column switching.

Fig. 3.

A comparison of bulky DNA adduct recovery utilizing two enrichment procedures, butanol extraction (top) and nuclease P1 treatment (bottom). In this study, oral mucosa DNA from tobacco chewers was investigated. The numbers indicate different subjects and are compared to a positive reference (right panels). Reprinted with permission from [1].

Fig. 4. The detection of bis(pentafluorobenzyl) PhIP hydrolyzed from rat lung DNA by GC-MS (A). B shows the deuterated (d_5) PhIP internal standard. Reprinted with permission from [38].

Fig. 5.

The detection of multiple BaP adducts from an in vitro reaction of BPDE with calf thymus DNA using CE-MS following sample stacking. Note the offscale signal for BaP-dGMP, the major adduct in the mixture as a result of the sample enrichment process. Reprinted with permission from [43].

Fig. 6.

One of the earliest examples of the use of capLC coupled with tandem QqQ-MS for the analysis of DNA adducts produced from the reaction of calf thymus DNA with N-acetoxy-AAF. The top panel shows the various AAF adducts detected when screening the digested reaction mixture by CNL. Fig. 7 (bottom) shows the dramatic sensitivity enhancement via the use of MRM for the specific transitions (473 $\rightarrow$ 357) m/z indicating the presence of AAF-dA adducts and (489 $\rightarrow$ 373) m/z for the AAF-dG adducts. Peaks denoted A and C are DNA adducts of interest in this study. Modified with permission from [40].

Fig. 7.
A comparison of ³²P-postlabeling and capillary LC–nanoESI–MS/MS of dG-IQ adducts from pancreatic tissue of non-human primates [27,54]. Reprinted with permission from [27].

Table 1

The DNA adduct detection methods discussed in this review highlighting the targeted adduct, the amount of DNA required, and the sensitivity of the method.

Platform	DNA Adduct	Sample req. (µg DNA)	Sensitivity (adducts/nuc.)	Ref.
³² P	AAP-dG	1	1/1e10	[19]
	dG-4-ABP	3	1/1e8	[38]
	IQ-dG	30	1/1e9	[64]
<i>GC-MS</i>				
	dG-4-ABP	100	0.32/1e8	[37]
	PhIP-dG	100	1/1e8	[38]
<i>LC-MS</i>				
	BaP-dG	100	0.3/1e8	[57]
	PhIP-dG	100	1/1e7	[55]
	BaP-dG	100	3/1e8	[30]
	MeIQx-dG	100	1/1e8	[56]
<i>microLC-MS</i>				
	IQ-dG	500	1/1e10	[54]
	AAF-dG	1000	1/1e6	[40]
	PhIP-dG	1000	3/1e8	[60]
	dG-4-ABP	300	5/1e9	[66]
	IQ-dG ^a	300	2/1e8	[63]
<i>nanoLC-MS</i>				
	IQ-dG	300	1/1e7	[61]
	IQ-dG	500	5.3/1e9	[54]
	BaP-dG	1000	2/1e8	[65]
<i>UPLC-MS</i>				
	Methyleugenol-dG	12.5	2/1e8	[76]
	dG-4-ABP ^b	10	1/1e7	[73]
<i>LC-MS column switching</i>				
	dG-4-ABP	100	0.7/1e7	[69]
	PhIP-dG	50	1.5/1e8	[70]
	dG-4-ABP ^c	5	5/1e9	[71]
	AA-I-dG ^d	10	0.3/1e8	[74]

^a MicroLC-nanoESI-MS.

^b MicroUPLC-MS.

^c Nano-LC-MS Column Switching.

^d MicroUPLC-MS Column Switching.