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Review

Methodologies for bulky DNA adduct analysis and biomonitoring of environmental and occupational exposures

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Abstract

It is undisputed that DNA adduct formation is one of the key processes in early carcinogenesis. Therefore, analysis of DNA adduct levels may be one of the best tools available to characterize exposure to complex mixtures of genotoxic chemicals as occurring in different environmental and occupational exposure settings. However, from an analytical point of view the detection and quantification of DNA adducts is a challenging enterprise as extremely high sensitivity and selectivity are required. The entire spectrum of chromatographic techniques, including thin-layer chromatography (TLC), gas and liquid chromatography as well as capillary electrophoresis has been used in combination with different detection systems, all with their own specific characteristics. Among the various combinations of techniques, the TLC–^{32}P-postlabeling combination appears to meet best with criteria of sensitivity and requirements of minimal amounts of material. Recent developments in the application of capillary electrophoresis in combination with either immunochemical or mass spectrometric detection techniques may offer new and promising approaches, with higher selectivity as compared to TLC–^{32}P postlabeling. The applicability of these new techniques in biomonitoring studies aiming at the exposure and risk assessment of low and chronic exposures remains to be determined. In this paper we compare and discuss the advantages and limitations of different techniques used in DNA adduct analysis, with specific emphasis on those adducts formed by the polycyclic aromatic hydrocarbons and heterocyclic aromatic amines.

Keywords: DNA

Contents

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1. Introduction

In our daily life we are continuously exposed to chemicals that may interact with cellular DNA to form DNA adducts. Unless these adducts are removed by DNA repair systems, they may lead to mutations as a consequence of misreplication. There is convincing evidence that mutations in relevant target sequences, such as oncogenes or tumor suppressor genes, are associated with the carcinogenic process [1]. The development of extremely sensitive analytical methods for detecting DNA adduct levels in the range of 1 per 10^8 to 10^{10} normal nucleotides, offers the opportunity to use DNA adduct measurements as a quantitative tool in exposure and risk assessment. The advantage of using DNA adduct measurements compared to environmental monitoring data in the context of risk assessment is that these early markers of effect integrate several aspects that determine the actual risk, including differences in exposure levels, absorption, distribution, metabolic (in)activation, genetic susceptibility and DNA repair capacity. On the other hand there is still debate on the biological significance of DNA adducts and the interpretation of adduct levels in terms of health risk [2,3]. There is however consensus that the use of adduct data in risk extrapolation has the greatest value when adduct structures have been characterized and the role of adduct removal and biological relevance of specific adducts are understood [2,4]. The presence of relatively high levels of certain endogenous DNA adducts, such as oxidized bases induced by endogenously formed oxygen radicals, may further complicate the interpretation of such data.

In this paper we review and discuss the analytical methods applied for the analysis of DNA adducts induced by environmental carcinogens. We focus on the analysis of bulky aromatic adducts, which can be correlated to environmental exposures based on their specific chemical structure, with particular emphasis on the polycyclic aromatic hydrocarbons and heterocyclic aromatic amines.

2. Formation of bulky aromatic DNA adducts

Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HCAs) are both classes of compounds that are formed by heating or incomplete combustion of organic material and which may form bulky aromatic DNA adducts. Environmental levels of PAHs have greatly increased as a consequence of industrialization, and numerous sources produce a mixture of 100–300 different PAHs, including PAHs with one or more NO_2 groups (nitro-PAHs) [5]. Many individual PAHs exert carcinogenic properties in experimental animals and in humans after conversion into reactive electrophilic metabolites by the oxidative enzymes, mainly cytochrome P450-related [6].

In 1974 Sims et al. reported the chemical synthesis of the 7,8-dihydrodiol-9,10-epoxide of B[*a*]P (B[*a*]PDE), which appeared to be the ultimate carcinogenic metabolite responsible for the binding to DNA [7]. Physicochemical studies showed that the highly reactive (±)-anti-B[*a*]P diolepoxide reacts mainly with guanine within DNA and polynucleotides; the C-10 carbon of B[*a*]P becoming linked to the exocyclic 2-amino group. Consequently, upon cellular replication, the binding of (±)-anti-B[*a*]P diolepoxide to genomic DNA may induce mutations in oncogenes or tumor suppressor genes and is likely to be a causative factor in several types of cancer [8].

Apart from the formation of PAHs during preparation or heating of food at high temperatures HCAs can also be formed. Under heating conditions from 150 up to 300°C, thermic mutagens are formed like IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine), classified as amino-imidazo-azaarenes [9]. Metabolic activation of heterocyclic amines to DNA reactive metabolites is hypothesized to occur via *N*-oxidation to *N*-hydroxy metabolites followed by *O*-acetylation to *N*-acetoxy arylamines [10]. The major adduct of PhIP is identified as *N*-(deoxyguanosin-8-yl)-PhIP. Similarly, the major adduct of IQ is the *N*-(deoxyguanosin-8-yl)-IQ adduct; 5-(deoxyguanosin-N^2-yl)-IQ is a minor adduct [11].

Environmental monitoring of, for example, PAHs in occupational environments or the analysis of PAHs and HCAs in food items or duplicate meals can provide information on potential exposures. However, in the context of risk assessment such an approach has its limitations because it does not take into account inter-individual variation in absorption, metabolism, excretion and bioavailability of the carcinogens. Since the formation of specific DNA

modifications appears to be a critical event in carcinogenesis, measurement of carcinogen–DNA adducts should provide biologically relevant information on the net result of exposure, absorption, metabolism, DNA adduct formation and DNA repair. Over recent years, the techniques employed for bulky DNA adduct detection and quantification have become well standardized and more reliable.

3. Methods for bulky DNA adduct determination

The assays for detection and quantification of DNA adducts need to be extremely sensitive since the lesions occur at very low levels. Much effort has been put into development of new, sensitive and specific immuno(cyto)chemical, biochemical and physicochemical techniques for DNA adduct detection. Over the last decades various techniques for detecting carcinogen–DNA adducts have been developed (see Table 1). These techniques can be generally classified into four major groups; (1) ^{32}P-postlabeling techniques; (2) immunochemical techniques; (3) fluorescence techniques; (4) mass spectrometric techniques.

3.1. ^{32}P-Postlabeling analysis

Traditionally, the detection of DNA adducts was achieved in experiments using a priori radiolabeled carcinogens and subsequent determination of the tracer in isolated DNA. The detection of adducts by postlabeling techniques involves the introduction of a radioisotope or other label into the adduct after it has been formed. In 1982, the first ^{32}P-postlabeling protocol for the detection of bulky aromatic compounds was published [12], and since then many researchers have used this assay for the detection and quantification of carcinogen–DNA adducts in human populations [13–17].

The ^{32}P-postlabeling method has been widely applied for DNA adduct detection because of its high sensitivity (1 adduct in 10^9 unmodified nucleotides) and that this sensitivity can be reached in small quantities of DNA (2–15 μg). The general outline of assay is as follows: (1) enzymatic digestion of DNA into 3′-mononucleotides; (2) an adduct enrichment procedure to enhance the sensitivity of the assay; (3) kinase-mediated phosphorylation of the adduct nucleotides with [γ-^{32}P]ATP of high specific radioactivity to form [5′-^{32}P]-3′-bisphosphates, and (4) chromatographic resolution of the labeled adducts (which are modified nucleoside 3′–5′-biphosphates) and their quantification. Originally, separation of adducts (step 4) was performed by ion-exchange thin-layer chromatography (TLC) in four directions. Subsequent quantification was achieved by scraping spots from the TLC plates, followed by liquid scintillation counting, but more recently this method is being gradually replaced by more modern plate scanning technologies. Several groups are also adapting high-performance liquid chromatography (HPLC) techniques, based on on-line detection of ^{32}P-radioactivity, instead of TLC to obtain better separation of postlabeled adducts [18]. By HPLC, the separation of adducts is considerably improved and very reproducible. Though from an analytical point of view, the resolution of HPLC is much better than of TLC, this methodology is still not sufficient for positive adduct identification. The disadvantage of HPLC with on-line radioactivity detection compared to TLC is the reduced sensitivity, which is possibly 10-fold less. Therefore, the HPLC method is limited for studies with high adduct levels, such as mechanistic studies applying cell lines or animal models [19], and seems less suitable for monitoring of human populations. Indeed, it has hardly been applied in field studies, and could not discriminate between PAH adduct levels in lymphocytes from smokers or non-smokers, whereas TLC could [20]. To improve the analytical capacity, the HPLC method can be used in parallel with TLC analyses of ^{32}P-postlabeled DNA adducts [21].

The current enrichment methods to enhance the sensitivity of the assay (step 2) are based on separation of adducts from normal nucleotides prior to labeling, or prevention of the latter from being labeled. The advantages and limitations of these methods have been discussed in several reviews [15,22–24]. In one procedure the DNA digest is extracted with butanol to isolate the hydrophobic adducts from the non-adducted nucleotides. An alternative approach uses the preferential dephosphorylation by nuclease P1 of normal 3′-nucleotides

Table 1
Characterization of different techniques applied for the analysis of bulky-aromatic–DNA adducts

Detection	Chromatography	Sensitivity adduct/10^8	Exposures	μg DNA needed	Cost	Throughput	Comments	Refs.
^{32}P-Postlabeling	TLC	0.1	Coal tar, ochratoxine A, PAH, styrene, tobacco, IQ, MeIQx, DiMeIQx, PhIP, unknown compounds/mixtures	2–10	Medium	Low	– Useful for screening variety of carcinogen adducts – Applicable to analysis of complex mixtures – Danger of underestimation of adduct levels due inefficient chromatographic recovery and phosphorylation – Interference of ''indogeneous'' spots	[13,14,70,78]
	HPLC	0.2–1.0	PAH, nitro-PAH PhIP	10	Medium	Low	– Less sensitive compared to TLC (±factor 10) but a relatively large amount of DNA can be analyzed to compensate the loss of sensitivity	[18,20,21,25]
	CE	0.1	B[*a*]P	10	Medium	Medium	– Enables multiple injections of a single sample	[35]
Immunochemical	Standard ELISA	1–4	Aflatoxins, 4-aminobiphenyl, cisplatin, coal tar, PAH including B[*a*]P, PhIP, DiMeIQx, oxidative damage, UV light	>100	Low	High	– Usual high specificity for carcinogen or class of carcinogens – Interference of substances that compete with antibody recognition	[39,41,79]
	CE	20	BPDE	0.5–2	Low	Medium	– Less DNA needed but also less sensitive	[45,46]
Fluorescence	HPLC	0.5–7	Aflatoxins, B[*a*]P, PAH, PhIP	>100	Low	High	– Only applicable to fluorescent compounds – Information on adduct identity needed	[57,58,60,63,80]
Mass spectrometry	GC/LC	0.3–10	4-Aminobiphenyl, PhIP, PAH, B[*a*]P, malondialdehyde, *N*-nitrosamines, NNK (tobacco)	>100	High	Low	– Structural identification of adduct – Derivatization needed	[23,62,63,65,70,71]
	CE	0.4–40	Styrene, phenyl glycidyl ethers BPDE	>100	High	Low	– Sample stacking used to lower detection limit	[71,75,77]
Accelerated mass spectrometry	HPLC	0.0026	MeIQx, PhIP	500	High	Low	– Not applicable in standard human biomonitoring studies due to use of ^{14}C-labeled substrates	[68,81]

compared to adducted 3′-nucleotides to enrich the sample in adducts. The dephosphorylated normal nucleotides are no longer substrates for kinase and will not be labeled in step 3. Both enrichment procedures are suitable for the analysis of PAH–DNA adducts, as they are relatively nuclease P1 resistant. However, some other adducts are partially digested by nuclease P1, and therefore not detected by this procedure. As, for example, HCA–DNA adducts are sensitive towards nuclease P1 digestion, further adjustments have been made to the standard butanol enrichment in order to improve the recovery of these adducts. Wohlin et al. optimized the butanol extraction by using butanol saturated ammonium formate (pH 3.5) to back extract adducted nucleotides [25]. Another approach to separate adducted and unadducted nucleotides before the labeling reaction is reversed-phase HPLC [26]. Alternatively, enrichment of adducts can be achieved by immunoaffinity chromatography (IAC) [27]. Over the years a number of antibodies has been raised against several classes of carcinogen–DNA adducts (reviewed in Ref. [28]). Since most antisera have a tendency to cross-react with DNA adducts of similar structure, predominantly structurally related DNA adducts are concentrated by IAC. Finally, there are studies in which an adduct enrichment step is completely omitted [29].

For the radioactive labeling of the adducts, generally two different methods are used. The standard method was described by Gupta et al. [12] and is based on the use of an excess of [γ-^{32}P]ATP over the substrate nucleotides. In this way, adducts and normal nucleotides become labeled quantitatively and to the same extent. The excess of ATP can be destroyed by adding potato apyrase after the labeling reaction has taken place. Alternatively, in the adduct intensification method [30] a limiting amount of carrier-free [γ-^{32}P]ATP is used. Because T4 polynucleotide kinase labels aromatic carcinogen–DNA adducts at higher rates than it does the normal nucleotides, these adducts become preferentially ^{32}P-labeled in this ATP-deficient method.

The covalent binding of a number of PAH to DNA in vivo has been analyzed by ^{32}P-postlabeling [15]. A complex PAH mixtures that binds to DNA produces a characteristic pattern of spots on poly(ethylenimine) (PEI)-cellulose TLC. A major drawback of the TLC–^{32}P-postlabeling method is the incapability to give conclusive information on structural identity of the detected DNA adducts. Even when an carcinogen–DNA adduct standard is available the resolving power of TLC is too limited to give definitive answers. The combined use of the ^{32}P-postlabeling method and HPLC demonstrated that the identification of PAH adducts formed by complex mixtures such as diesel exhaust extracts is considerably improved [31]. Some years ago, mass spectrometry (MS) in tandem with ^{32}P-postlabeling was used to provide positive identification of carcinogen–DNA adducts in human samples [23]. In future, further advancements in adduct characterization is expected by applying sophisticated separation procedures for adducts as HPLC and capillary electrophoresis (CE).

Contradictionary, detection of unidentified adducts is one of the strengths of the postlabeling assay since this enables the possibility to detect multiple hydrophobic adducts after exposure to complex mixtures of unknown sources. However, with unknown samples absolute quantitation becomes more difficult because conditions for digestion, enrichment and labeling cannot be optimized for each adduct. Furthermore, quantitation can be hampered by incomplete hydrolysis of DNA leading to the appearance of additional spots in postlabeling analysis, due to the presence of adducted oligonucleotides. In order to achieve complete hydrolysis of HCA adducts, an extra enzymatic digestion step can be carried out after the labeling reaction. The enzyme used for this purpose may be nuclease P1 [32] or phosphodiesterase I [33]. Moreover, even a combination of both enzymes can be used [34]. Obviously, incomplete hydrolysis may lead to errors in quantitation. Finally, the efficiencies of nuclease P1 reaction (adduct enrichment) and phosphorylation may differ from adduct to adduct. Consequently, erroneous adduct values may be found when assaying a certain adduct under suboptimal conditions, especially when proper standards with known adduct levels are not available and assayed in parallel. These issues of validation and standardization have been addressed in interlaboratory trials [22,24]. Within these studies several laboratories analyzed the same samples using their own optimized protocols and a consensus protocol. The first trial demonstrated remarkable differences in

absolute adduct levels, indicating that values obtained from different laboratories should be compared with caution [22]. Evaluation of all critical aspects that might contribute to this variability, including DNA hydrolysis, enrichment procedures, nuclease P1 digestion, labeling, separation and quantitation, resulted in a refined standard protocol, presented in detail by Phillips and Castegnaro [24]. The use of this protocol showed a 20% reduction in variability of PAH adducts as compared to the first trial. Nevertheless, discrepancies between estimates by ^{3}H incorporation and ^{32}P-postlabeling indicate that true adduct levels are yet difficult to establish. Moreover, these trials showed that optimum conditions for the reproducible quantitation of PhIP–DNA adducts remain to be assessed. Recently, yet another new technique for the detection of ^{32}P-postlabeled DNA adducts was developed, based on separation by capillary zone electrophoresis [35]. The advantage of this approach is the combination of the high selectivity of capillary electrophoresis with a sensitive on-line blotting method. The eluted radioactive peaks are directly transferred from the capillary outlet to a positively charged moving filter paper, followed by quantification using a phosphor imaging detector. Adduct levels of 1 per 10^{9} unmodified nucleotides have been reported. Further advantages of this system, compared to TLC separation, are the possibility to carry out multiple injections from a single sample and the reduction of analysis time.

3.2. Immunochemical methods

Immunoassays to determine DNA adducts are based upon eliciting and characterizing polyclonal and monoclonal antisera against these haptens. Several reviews on immunochemical detection of carcinogen–DNA adducts are available [36–38]. Different types of competitive immunoassays have been used for adduct measurement and generally quantitation is based upon competition of the antibody–antigen binding by antigen in the sample. For instance, to quantify PAH–DNA adducts competitive enzyme-linked immunosorbent assays (ELISAs) using antisera recognizing BPDE-modified DNA have been used frequently [39,40]. Monoclonal antibodies were also raised against several heterocyclic amines and their DNA adducts in order to make specific and sensitive detection and purification systems suitable for biological samples [41]. As a screening device immunoassays have main advantages such as the relative straightforwardness to perform, the inexpensiveness, and a high throughput. The detection limit depends on affinity of the antisera against a certain adduct but sensitivity of 1–4 adducts in 10^{8} unmodified nucleotides can be reached under optimal assay conditions. However, quantitative immunoassays have the disadvantage that they require large amounts of DNA (100 μg). An additional disadvantage of immunoassays is that a priori not only the chemical structure of the adduct must be known but also the adduct must be available in sufficient quantities to raise the specific antibodies in mice or rabbits. Normally, the affinity of antisera is highest for the original adduct used for immunization, but cross-reactivities with chemically related structures are commonly observed. For instance, antisera against BPDE–DNA recognize other PAH diol epoxide-modified DNAs, putatively those from PAH undergoing ''bay region'' activation. Consequently the chemical specificity of the assay is low and absolute quantitation of BPDE–DNA adducts is hampered when DNA is analyzed from subjects exposed to complex mixtures of PAH. In the case that assays are calibrated with BPDE-modified DNA standards adduct levels are expressed in BPDE–DNA equivalents [40]. Several efforts have been made to increase the sensitivity of the competitive ELISA and/or lowering the amount of DNA required [42,43].

Alternatively, non-competitive immunoassays have been developed in quantitation of adducts including BPDE–DNA adducts. Originally, the sensitivity of these assays was hampered by the limited amounts of DNA that can be immobilized quantitatively to the plastic surface of ELISA plates. Immuno-slot blot assays were developed to overcome this problem; immobilization of DNA on nitrocellulose filters (0.5–1 μg DNA/mm^{2}) is higher compared with microtiter plates (20–30 ng/well) [39]. However, a non-competitive immuno-slot blot assay developed for the detection of BPDE–DNA adducts

reached only a sensitivity of 0.2 adducts/10^6 nucleotides [44].

By combining immunochemical recognition with capillary electrophoresis and laser-induced fluorescence, Le et al. [45] demonstrated the possibility to detect DNA damage at the zeptomole (10^{-21}) level. Compared to the absolute level of detection of the ^{32}P-postlabeling assay, being about 10^{-15} to 10^{-17}, this is extremely sensitive. This assay that was first developed and applied for the detection of thymine glycol, has also been used for other forms of DNA damage, including BPDE [46]. This assay requires only 0.5 to 2 μg DNA per sample, considerably less as compared to other immunoassays, but appears to be less sensitive as compared to the postlabeling assay in terms of detectable relative adduct levels (0.2 adducts per 10^6 normal nucleotides). This limit of detection might be lowered another order of magnitude, but that would in turn require the use of more DNA.

In addition to their use in quantitative immunoassays, specific antibodies against carcinogen–DNA adducts have been used in immunocytochemical staining of tissues and cells (reviewed in Refs. [47,48]). Recent progress in staining procedures, more sensitive endpoints, and quantitation with computer-assisted technology has made this assay accurate to quantitatively distinguish samples [49–51]. Polyclonal antibodies were also raised against PhIP–DNA adducts for their immunohistochemical demonstration in paraffin-embedded sections [52,53]. Although immunocytochemistry has a much lower sensitivity (1 adduct in 10^6 unmodified nucleotides) than the ELISA, it has the great advantage that localization of carcinogen–DNA adducts at the individual cellular level can be studied.

3.3. Fluorescence techniques

HPLC with fluorescence detection (FL) has also been developed for the determination of DNA adducts [54–56]. The HPLC–fluorescence assay has been optimized for the quantitation of levels of the biologically most active (+)-anti-B[*a*]PDE–DNA adduct through chemical hydrolysis of the respective B[*a*]P-tetrols [57]. This assay has a detection limit of one B[*a*]P adduct in 10^8 unmodified nucleotides, and could measure adducts at a level of up to 4–10 in 10^8 nucleotides in white blood cells. In this study a significant association was found between the B[*a*]P–DNA adduct measurements by fluorescence and ^{32}P-postlabeling ($r=0.95$; $P=0.001$). A comparative study with B[*a*]P exposed rats showed a detection limit for HPLC–FL analysis varying from 0.5 to 7.4 adducts per 10^8 nucleotides, while for postlabeling this was around 1 adduct per 10^9 nucleotides [58]. The HPLC–FL assay can be used to identify BPDE isomers with different biological effects and might therefore be of value in exposure and risk assessment of individuals exposed to PAHs. Furthermore, the assay was found to be sufficiently sensitive to detect BPDE–DNA adducts in coke over workers [59]. Marsch et al. [60] used a fluorescence spectroscopic method to characterize PhIP–DNA adducts synthesized in vitro, rather than measuring adduct levels in a biomonitoring setting.

Overall, the major limitations of the use of fluorescence spectroscopy for the detection of DNA adducts in tissues are that prior knowledge of the chemistry of the adducts concerned is needed and the requirement that the adduct is intrinsically fluorescent. Additional disadvantage is that to reach sensitivity large quantities of sample DNA (100–1000 μg) are necessary. An advantage is that assays can be performed rapidly and inexpensively, once the initial costs of the equipment has been made.

3.4. Mass spectrometric analysis

Gas chromatography–mass spectrometry (GC–MS) is highly specific and has been widely applied in the measurement of carcinogen–protein adducts [61] and to a lesser extent, of carcinogen–DNA adducts [23,62–66]. The major advantage of GC–MS as compared to other techniques is that it provides information on molecular mass and structure of the adduct, and thus contributes to the positive identification. Furthermore, it can help to identify unknown adducts and their structures, which may be of importance to explain working mechanisms of the genotoxic agents under study.

Generally, this technique needs derivatization of

the adduct to increase mass and volatility, vaporization of the sample; ionization, which can be achieved in a number of ways (electron impact, fast atom bombardment, chemical ionization, and laser desorption); collimation of the charged particles; and acceleration into the mass analyzer. The spectrum of ions detected comprises the molecular ion plus the fragment ions including the base peak. The base peak, the most intense signal, is often used for quantitative sample analysis by single-ion monitoring when the mass spectrum of a compound of interest is already known.

Methods using GC–MS are available for certain exposures mainly related with tobacco smoking, such as 4-aminobiphenyl (4-ABP) and 4-(methylnitrosoamine)-1-(3-pyridyl)-1-butanone (NNK), or dietary intake (including IQ and PhIP) (see Table 1). For instance, the presence of BPDE-dG has been demonstrated by GC–MS in placental tissue of smokers and nonsmokers [67]. Furthermore, PhIP–DNA adducts (35–135 adducts/10^{12} nucleotides) have been detected in normal human colon tissue after exposure to a dietary-relevant dose of the carcinogen, using the very sensitive accelerator mass spectrometry (AMS) method [68]. The limit of detection for this technique is in the order of a few adducts per 10^{12} nucleotides [69]. However, AMS is not applicable in standard human biomonitoring studies as it depends on the use of ^{14}C-labeled substrates. Alternatively, GC–MS has been applied to confirm the identity of PhIP–DNA adducts quantified by ^{32}P-postlabeling analysis in human colon [70]. Also LC–MS can be used to measure PhIP–DNA adducts, with both techniques having the same limit of detection [71]. Though several GC–MS methods have been developed, so far applications for carcinogen–DNA adduct measurements in biological matrices have been limited, partly because of the great expenses and the relatively large amount of sample needed for analysis to reach sensitivity.

Combined liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) has been applied to analyze DNA adducts at the nucleotide level without prior derivatization. This allows the study of sugar–phosphate modifications that is not possible with GC–MS [72]. Subsequently, the sensitivity was improved by combining it with column switching techniques and nanoscale liquid chromatography, which also allowed on-line sample cleanup by removing the unmodified 2′-deoxynucleotides [73]. Such a NanoFlow ESI LC–MS system, improved the mass sensitivity by a factor of 3300, and demonstrated different DNA base- and phosphate-alkylated adducts of bisphenol A diglycidyl ether. Further experiments will have to prove the value of this technique in the in vivo situation.

The combination of capillary zone electrophoresis (CZE), as a high efficiency separation technique, and electrospray tandem mass spectrometry (ES-MS–MS) has proven to be very promising in the field of DNA adduct research. So far, this combination has been applied for the analysis of adducts formed by several different types of compounds, including BPDE [74], styrene oxide [75] and phenyl glycidyl ethers (an important class of industrial epoxides) [76,77]. A clear limitation of CZE is the relatively small sampling volume (1–10 nl). Sample stacking with solvent removal is one of the techniques that have been developed in order to increase the sampling volume, and thus to lower the detection limit. This technique results in a 1000-fold pre-concentration without significant loss in resolution [78]. In combination with solid-phase sample cleanup, adduct levels of four BPDE adducts in 10^{7} unmodified bases can be detected [73].

4. Concluding remarks and future perspectives

The development of a variety of methodologies for measuring different classes of DNA adducts has produced useful tools for exposure assessment of genotoxicants that may also serve as biomarkers of very early genotoxic effects in the environmentally exposed humans. For an assay to be applicable in low and chronic exposure settings it must meet a set of common criteria: (1) sensitive enough to detect low levels of adducts, (2) requirement of only micrograms of DNA when limited amounts of tissue are available, (3) giving results quantitatively related with exposure, (4) applicability to unidentified adducts that may be formed after exposure to complex mixtures with unknown chemical composition, (5) capacity to resolve, quantitate and identify adducts,

and (6) as a screening device the ability to assay a number of samples in a short period. Among the present techniques, the ^{32}P-postlabeling seems to best meet these criteria overall. Especially, the sensitivity and applicability on DNA adducts induced by unknown environmental chemical mixtures have made the ^{32}P-postlabeling assay favorite by most investigators and most studies have been using this assay. On the other hand, the assay is time-consuming and requires large quantities of radioactivity. In this context, the use of capillary electrophoresis appears to be promising, as it reduces analysis time considerably. At present, however, there is insufficient experience with this methodology, particularly in human biomonitoring studies, to come to final conclusions.

As a screening instrument, immunoassays might be appropriate in situations where tissue availability and sensitivity is less critical. Strong arguments in favor are the relative ease to perform the ELISA and low laboratory investments, for instance only a microtiter reader is needed. Although the ELISA might reach the sensitivity needed in relatively high (occupational) exposure situations, many human biomonitoring studies are aimed at the risk assessment of low and chronic exposures. Furthermore, the amount of DNA needed can only be obtained in limited human exposure studies, and not in larger ambulant populations. It is not yet clear whether or not further development of CZE, combined with immunochemical techniques, will allow sufficiently sensitive detection of DNA adducts, without the use of the same amounts of DNA as in the standard ELISA.

HPLC–FL can be applied in biomonitoring studies of BPDE–DNA adducts but has its limitations in exposure assessment of complex PAH mixtures in human populations, as it requires relatively high amounts of material. At present, GC–MS is less attractive to be used as a screening method because of the high expenses and the little amount of work done in the field of biomonitoring of environmental exposures.

It has become clear that researchers in the field of environmental epidemiology should be aware of the advantages and limitations of each of the techniques available for DNA adduct measurement. Based on this knowledge, it should be possible to select the most appropriate assay in order to achieve the research goals of a particular study.

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