University of Alberta

Immunoelectrophoretic Detection of DNA Adducts in White Blood Cells

by

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ABSTRACT

A fluorescence immunoassay with capillary electrophoresis and laser induced fluorescence (FIA/CE/LIF) technique was developed and tested suitable for the detection of benzo(a)pyrene-diol-epoxide (BPDE) DNA adducts in mononuclear white blood cells (MNCs). The developed method was tested for both *in vitro* and biomonitoring applications. Firstly, *in vitro* exposure of MNCs to both BPDE and buthionine sulfoximine, a glutathione depleting agent, resulted in an increase in adducts compared to BPDE exposure alone. In carrying out this experiment, we confirmed the important role of glutathione in BPDE inactivation in MNCs, and validated the use of FIA/CE/LIF for in vitro studies of BPDE-DNA adducts. To test the suitability for human biomonitoring, FIA/CE/LIF analysis was performed on MNCs of smokers. The adducts were detectable, however not quantifiable. Further improvements to sensitivity, as well as assessment of adducts in MNCs from other smokers, will help to identify whether FIA/CE/LIF is an appropriate technique for human biomonitoring of BPDE-DNA adducts.

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LIST OF ABBREVIATIONS

1°-Ab	Primary antibody
2°-Ab	Secondary antibody (fluorescently labeled antibody)
AMS	Accelerated mass spectrometry
BaP	Benzo(a)pyrene
BPDE	Benzo(a)pyrene-diol-epoxide
BPDE-dG	BPDE-N^2-deoxyguanosine
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CE	Capillary electrophoresis
CIA	Chemiluminescence immunoassays
ELISA	Enzyme-linked immunosorbent assays
EPF	Electrophoretic force
EOF	Electroosmotic force
ESI	Electrospray ionization
FIA	Fluorescence immunoassays
FIA/CE/LIF	Fluorescence immunoassay with capillary electrophoresis and laser induced fluorescence
GC	Gas chromatography
γ-GCS	γ-glutamylcysteine synthetase
GSH	Glutathione
GST	Glutathione-S-transferase
HPLC	High performance liquid chromatography
i.d.	Inner diameter
IgG	Immunoglobulin G
LC	Liquid chromatography
LIF	Laser-induced fluorescence
LMPCR	Ligation-mediated polymerase chain reaction
MNC	Mononuclear white blood cell
MS	Mass spectrometry

PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PMT	Photomultiplier tube
RBC	Red blood cell
RIA	Radioimmunoassays
RPA	Relative peak area
SE	Standard error
TG	Tris-glycine
TLC	Thin layer chromatography
TMR	Tetramethyl rhodamine

Chapter 1
Introduction

1.1 Benzo(a)pyrene

Benzo(a)pyrene (BaP) is a carcinogenic polycyclic aromatic hydrocarbon (PAH) formed via the incomplete combustion of organic substances [1]. Sources of BaP include cigarette smoke, coal burning, automobile exhaust, and smoked foods. It is also found in tar and asphalt, as well as from natural sources such as forest fires, grass fires, and volcanic eruptions [2]. As BaP is produced from a variety of sources, it is widespread in the environment. Due to BaP's low water solubility, low vapor pressure, and high octanol-water partition coefficient, environmental BaP persists in soil and sediment [1,3,4]. A small percentage of BaP partitions into air, water, and biota.

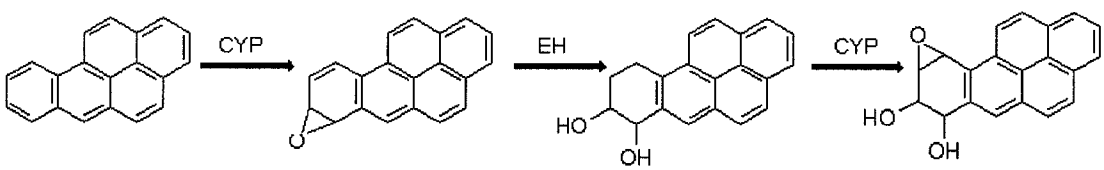
Human exposure can occur via ingestion of smoked foods, cigarette smoking, and inhalation of contaminated air [5]. The primary source of BaP exposure in non-smokers is food, which accounts for 97-99% of total BaP exposure [1,3]. The concentration of BaP in foods will vary with the type of food, surrounding environment, and the method of cooking. Minor BaP exposure is attributed to air, water, and soil; the BaP concentrations will vary considerably depending on the season, rural or urban location, and surrounding industry. The average total daily intake of BaP has been estimated to be 2.2 μg/day; however, this value may be elevated in heavy smokers [3].

The International Agency for Research on Cancer recently changed the classification of BaP from a probable human carcinogen (Group 2) to a known human carcinogen (Group 1) [6]. BaP is considered to be a cause of lung, skin, and bladder cancers in humans [7]. Once BaP is taken up in the body, it is converted to the highly reactive benzo(a)pyrene-diol-epoxide (BPDE); as shown in Figure 1.1a, epoxide hydrolase and cytochrome P450 enzymes are responsible for this conversion [8]. The epoxide group of BPDE is highly electrophilic and can covalently bind to nucleophilic regions of DNA. This leads to the formation of BPDE-DNA adducts. The major BPDE-DNA adduct, BPDE-N^2-deoxyguanosine (BPDE-dG), is formed when the epoxide group of BPDE covalently binds to the 2-amine position of a guanine base (Figure 1.1b) [9].

BPDE-DNA adducts may cause genetic mutations and/or cause chromosomal instability. For example, BPDE-DNA adducts have been known to cause GC→ TA transversions [10]. If the adduct or the resultant DNA damage is not repaired, this may potentially cause chemical carcinogenesis.

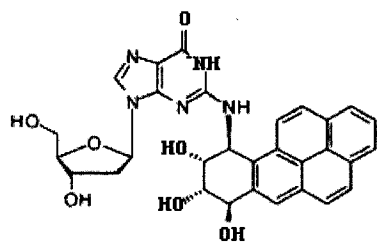
As BPDE-DNA adduct formation is an important step in BaP carcinogenicity, the analysis and characterization of BPDE-DNA adducts may help to improve cancer risk assessments. This thesis focuses on the development of a new method for BPDE-DNA adduct analysis in hopes of improving the sensitivity and specificity of currently used methods.

(a)



Benzo(a)pyrene | Benzo(a)pyrene 7,8 epoxide | Benzo(a)pyrene 7,8 diol | Benzo(a)pyrene 7,8 diol-9,10 epoxide (BPDE)

(b)



BPDE-N^2-deoxyguanosine

Figure 1.1 (a) The major metabolic pathway of benzo(a)pyrene leading to the ultimate carcinogen, benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE). CYP, cytochrome P450; EH, epoxide hydrolase. (b) Structure of BPDE-N^2-deoxyguanosine, the major BPDE-DNA adduct. [8]

1.2 Biomarkers

The US National Research Council defines biological markers as indicators that signal events in biologic systems or samples [11]. They are used to clarify the relationships between xenobiotic exposure and health impairment. Biological markers, or biomarkers, can be measured in body fluids, cells, or tissues, with blood and urine being the most common specimen choice. Biomarkers are generally categorized into three types: markers of exposure, markers of effect, and markers of susceptibility.

1.2.1 Biomarkers of Exposure

A biomarker of exposure is an exogenous chemical, its metabolites, or the product of an interaction between the chemical and a target molecule or cell [12]. Biomarkers of exposure are the most useful and most common marker for environmental health research. They provide reasonable estimates of individual exposure, taking into account all routes of exposure and inter-individual variations in absorption, distribution, metabolism and excretion [13]. Within the category of biomarkers of exposure, a further classification can be made into markers of internal dose and markers of biologically effective dose. Markers of internal dose represent the amount of xenobiotic that has entered the body of an individual, and include measures of the parent compound (eg. lead in blood [14]) or its metabolites (eg. urinary cotinine, a metabolite of nicotine [15]). Markers of the biologically effective dose indicate the amount of material that has interacted with the critical cellular macromolecules [11]. Examples include DNA

adducts, such as aflatoxin B_1-DNA adducts in urine [16], and protein adducts, such as 4-aminobiphenyl-hemoglobin adducts in blood [17].

1.2.2 Biomarkers of Effect

A biomarker of effect is a measurable biochemical, physiological, or other alteration in a biological system resulting from exposure, that is predictive of health impairment or potential health impairment [12]. Biomarkers of effect represent functional or structural changes to organs or tissues. Examples include retinol binding protein in urine following exposure to cadmium [18], and γ-aminolevulinic acid in plasma or urine following exposure to lead [14]. DNA adducts may also provide an indication of early biological effects.

1.2.3 Biomarkers of Susceptibility

A biomarker of susceptibility is an indicator of an inherent or acquired property of an organism that causes an increase in the internal dose of a xenobiotic or an alteration in the response to a xenobiotic [19]. Variations in the absorption, distribution, metabolism, excretion, and/or DNA repair of an environmental xenobiotic may be responsible for differing individual responses to a particular dose. For example, there is a higher incidence of bladder cancer following exposure to aromatic amines in individuals possessing a less functional form of the N-acetyltransferase enzyme [20]. Thus, the phenotype of N-acetyltransferase is a biomarker of susceptibility.

1.2.4 Biomarkers in Risk Assessment

Biomarkers can provide a measure of individual exposure or effect to a particular environmental chemical. Because biomarkers take into account different exposure levels, all routes of exposure, and varying individual responses, biomarkers are a valuable addition to environmental risk assessment [21].

Risk assessments have typically been based on data obtained from human epidemiology studies and animal toxicology studies, and both have their merits and disadvantages. Although some animals can be informative models for human health research, there is still much uncertainty in the extrapolation to humans. In addition, animal studies are generally based on exposures to high doses of a chemical, and then extrapolated to apply to the low doses observed in the environment; this adds more uncertainty into the analysis. Epidemiological studies may be able to reveal associations between exposures and outcomes, but they are expensive, time-consuming, have confounding factors, and are usually only applicable to high-exposure situations. In addition, the determination of exposure has always been a primary weak point in epidemiological studies. Exposure assessments based on interviews and/or self-reported questionnaires is a potential source of bias and misclassification in many studies [22]. Also, using environmental levels as the exposure variable is not entirely accurate, as it does not take individual variation into consideration.

To increase the confidence in health risk assessments, it is necessary to improve the existing exposure assessment methods. Environmental monitoring is currently the most common approach to determining exposure. However, biological monitoring, when practicable, is more valuable than environmental monitoring to determine level of risk [23]. Thus, biomarkers are a suitable source for determination of environmental exposures, and the use of DNA adducts as biomarkers in carcinogen exposure assessments may be extremely beneficial for health risk analyses.

1.3 DNA Adducts as Biomarkers

DNA adducts are the products of covalent interactions between chemical carcinogens and DNA. The adducts may lead to DNA shape distortions, cause point mutations, modify replication, and/or cause chromosomal instability. These genetic modifications have the potential to become permanent mutations, which can lead to the development of cancer. Because DNA adducts may be an integral part of cancer initiation, DNA adducts as biomarkers have the potential to provide a useful means for estimating exposure and cancer risk.

1.3.1 Chemical Carcinogenesis

Chemical carcinogenesis is a multistage process comprised of three stages: initiation, promotion, and progression [24]. The initiation step is characterized by a genotoxic event occurring in a cancer-related gene (e.g., tumor-suppressor gene, proto-

oncogene, DNA repair gene, apoptosis-related gene) following exposure to a genotoxic agent. The genotoxic agent may be a direct-acting carcinogen (eg. ionizing radiation), or a pro-carcinogen (eg. BaP), which requires metabolic activation to its ultimate carcinogenic form. If the DNA damage is not repaired, and cell death does not occur, the cell is considered an initiated cell. The initiated cell may remain dormant, and not cause any harm to the individual. Alternatively, the cell may undergo clonal expansion into a benign neoplasm. This step in carcinogenesis is called promotion, and occurs under the influence of one or more promoting agents. Promoting agents (eg. phorbol, testosterone, croton oil [25-27]) enhance the growth of initiated cells, but may not necessarily directly cause DNA damage. Subsequent genotoxic events characterize the progression stage of carcinogenesis. Additional critical mutations to the benign neoplastic cells may alter the existing growth pattern of the cell, resulting in growth of the benign neoplasm into malignant neoplasm.

1.3.2 Carcinogen Exposure, DNA Adducts and Cancer

Damage to DNA is a key initial step of chemical carcinogenesis. One possible mechanism for environmental carcinogens to cause DNA damage is through the formation of DNA adducts. The detection and quantification of specific DNA adducts can provide an estimate of the biologically effective dose of a carcinogen. An important factor in the use of DNA adducts as biomarkers is the relationship between carcinogen exposure, DNA adduct formation, and carcinogenesis [28]. The process of validating DNA adducts as biomarkers is complicated; multiple studies of different types must be

done to identify and verify the role of DNA adducts in the carcinogenesis pathway [19]. Animal models, *in vitro* assays, and epidemiologic studies are the primary methods used to elucidate this pathway.

1.3.3 BPDE-DNA Adducts as Biomarkers

An important study providing evidence of an etiological link between BPDE-DNA adducts and cancer was published by Denissenko et al [29]. They analyzed the distribution of BPDE-DNA adducts along the *p53* tumor suppressor gene in cancerous HeLa cells and normal bronchial cells. Major mutational hotspots in human lung cancers are in codons 157, 248, and 273 of the *p53* gene. Following cellular exposure to BPDE, they found that selective BPDE-DNA adduct formation occurred at guanine positions in the same three codons of the p53 gene in both cell types. The co-incidence of mutational hotspots and adduct hotspots suggest that BPDE-DNA adducts are involved in the initiation of human lung cancer. This study provides evidence of the intermediary role of BPDE-DNA adducts in carcinogenesis, and supports the use of BPDE-DNA adducts as biomarkers. The relationship between BPDE-DNA adducts and BaP exposure has been extensively studied in animals and humans. The association between BPDE-DNA adducts and cancer development has been more difficult to identify, however the evidence does appear to support that DNA adducts are related to cancer risk [21].

1.3.3.1 BPDE-DNA Adducts and BaP Exposure

Many studies have shown that exposure to BaP in test animals results in increased DNA adduct formation [30-32]. Dunn observed a correlation between dose and adduct formation in the stomach and liver of mice following oral BaP exposure [33]. This study was among the first to show a linear relationship for BaP adduct formation. Talaska et al. also examined the relationship between BaP exposure and adduct formation in mice [34]. They administered low doses of BaP to the skin of mice, and measured the levels of BPDE-DNA adducts in the skin, lung, and liver. Adduct levels in the skin and lungs increased with increasing exposure, however the skin showed a much higher level of BPDE-DNA adducts than the lungs. They concluded that the different tissue responses were due to the variations in tissue levels of bioactivation and DNA repair enzymes. Others have also observed varying tissue responses following BaP exposure in mice [35,36].

A number of epidemiologic studies have assessed the correlation between BaP exposure and BPDE-DNA adducts. Shamsuddin et al. were among the first to identify the formation of BPDE-DNA adducts in occupationally exposed humans [37]. Hemminki et al. found that BPDE-DNA adduct levels in blood of coke workers and residents around the cokeries were higher than rural controls; however, there was no correlation between ambient BaP concentration and adducts [38]. Other studies have confirmed the presence of BPDE-DNA adducts in occupationally exposed individuals[39-42]. Rothman et al. found that DNA adducts in blood of firefighters were

associated with consumption of charbroiled meats, but not hours of firefighting duty [43]. This finding is supported by the observation that 97-99% of BaP exposure comes from food [1,3].

1.3.3.2 BPDE-DNA Adducts and Carcinogenesis

The role of BPDE-DNA adducts in cancer initiation has not been fully uncovered. Several animal studies have shown a relationship between adducts and tumor initiation [44-48]; however, other animal studies have found the relationship to be very complex, and that DNA adduct levels were not predictive of carcinogenesis [21,49]. Though the results of animal studies are inconclusive, recent epidemiology studies have supported the association between adducts and cancer. A recent case-control study identified that smokers with elevated levels of DNA adducts in white blood cells were up to 3 times more likely to develop lung cancer [50]. Peluso et al. identified a strong correlation between DNA adducts in white blood cells and risk of bladder cancer [51]. Additionally, Li et al. observed an association between the level of induced BPDE-DNA adducts in lymphocytes and risk of lung cancer [52]. The conflicting results between animal studies and epidemiology studies represents a current knowledge gap regarding the carcinogenic predictiveness of BPDE-DNA adducts.

1.3.4 Limitations of DNA Adducts as Biomarkers

In using DNA adducts as biomarkers, several limitations must be considered. Of principal concern is the significant interindividual variability in the formation of carcinogen-DNA adducts. This biological variation stems from differences in carcinogen absorption, distribution, metabolism, and excretion, as well as differences in DNA repair. There is also individual variation in the amount of endogenously formed DNA adducts, and the background levels of tumors. These differences make it difficult to establish human dose-response associations, and complicates the assessment of individual risk based on the presence of DNA adducts.

It is important to thoroughly characterize the DNA adducts formed by the carcinogen of interest. A single carcinogen may form many different adducts; for example vinyl chloride can form 7-(2-oxoethyl)guanine, N^2,3-ethenoguanine and 1,N^6-ethenoadenine [53,54]. Also, a single adduct can be formed by multiple carcinogens; for example 8-hydroxy-2'-deoxyguanosine can be formed from asbestos, hydrazines, and 4-aminobiphenyl [55-57]. Also, the DNA adducts formed endogenously may be identical to those formed by carcinogens, further complicating the characterization of DNA adducts.

Another issue relates to the occurrence of DNA repair and cell death, as these processes are involved in the removal of DNA adducts. The level of measured DNA adducts at any point in time is reflective of the rate of DNA adduct formation and DNA

repair, and the extent of cell death. Additionally, cell turnover may cause dilution of DNA adducts. Therefore, it is important to consider the timing of sampling in any study, as these factors may have a significant effect on the amount of measured DNA adducts. It is necessary to understand that the measured level of adducts does not represent permanent DNA damage.

Furthermore, the multifactorial origin of cancer makes it difficult to clearly define what causes a cancer. The presence of carcinogen-DNA adducts does not necessarily indicate that a cancer will develop, as not all adduct formation leads to carcinogenesis. In most cases, it is not known to what degree the biomarkers predict the risk of mutation or cancer, which limits the application of DNA adducts in risk assessment [58]. Thus, thorough characterization of individual carcinogen-DNA adducts is required prior to their use as biomarkers of exposure and as early indicators of potential disease.

1.4 Techniques for Measuring BPDE-DNA Adducts

Accurate and sensitive DNA adduct detection methods are essential for the use of DNA adducts as biomarkers. Several methods have been developed to detect BPDE-DNA adducts in human tissues, blood, and urine, and these methods can be used to qualitatively and/or quantitatively measure presence of DNA adducts. Among the most common techniques are ^{32}P-postlabelling, immunoassays, and mass spectrometry [59].

More recent techniques include gene specific or sequence specific DNA adduct detection methods.

1.4.1 ^{32}P-postlabelling Assay

The ^{32}P-postlabelling assay, which was first developed in the early 1980's, involves four major steps (Figure 1.2) [60,61]. Firstly, DNA is digested into component nucleotides by endo- and exo-nucleases. Secondly, a radiolabelled phosphate group is incorporated into each nucleotide by addition of [γ-^{32}P] ATP + T4 polynucleotide kinase. Next, separation of the normal nucleotides from the adducts is performed using cellulose thin layer chromatography (TLC), or high performance liquid chromatography (HPLC); for most carcinogens, the DNA-adducts are hydrophobic, and thus can be separated from the less hydrophobic normal nucleotides. The last step involves the detection of ^{32}P radioactivity.

The ^{32}P-postlabelling assay offers versatility, as adducts from most chemical classes of carcinogens can be monitored, and no prior knowledge of the adduct structure is needed. It has the ability to screen for carcinogen-DNA adducts without the initial labelling of the carcinogen with a radioactive isotope; this provides a feasible and cost-effective method for carcinogen-DNA adduct measurements throughout long-term animal studies because the carcinogen does not need to be radiolabelled prior to administration. Other advantages include high sensitivity (1 adduct in 10^9 nucleotides), and small sample requirements (2-10 ug DNA) [20, 62]. Weaknesses of this assay include limited

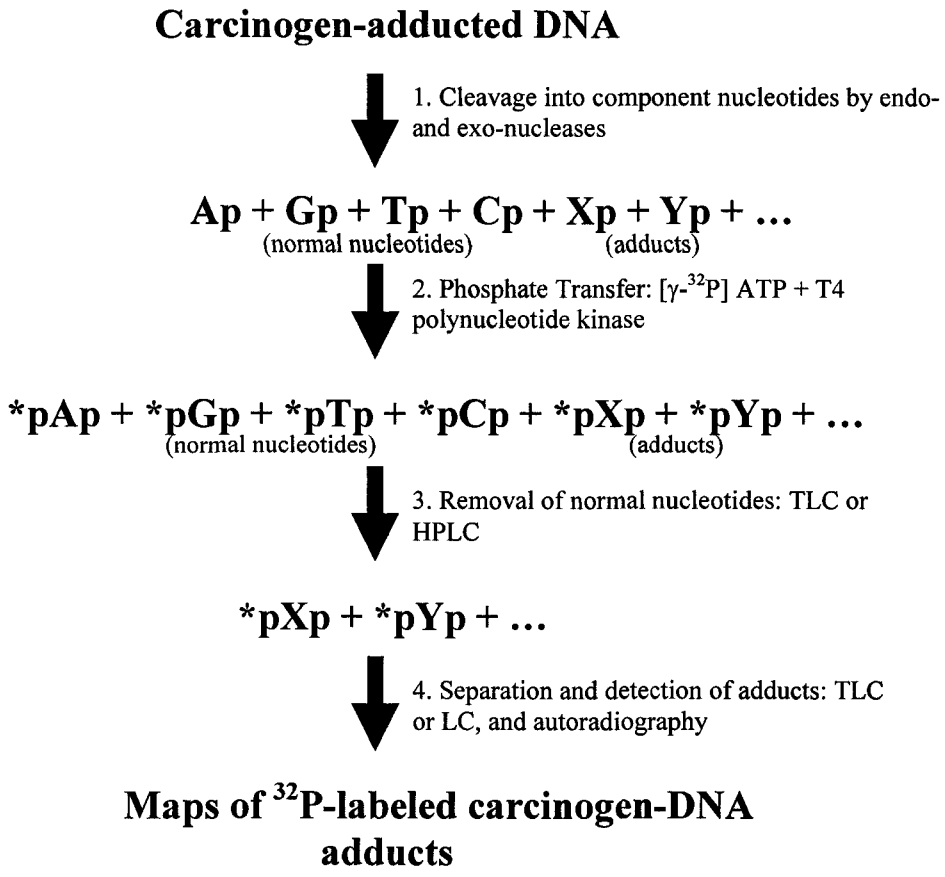


Figure 1.2 Major steps of the ^{32}P-postlabelling assay. Asterisks indicate positions of ^{32}P label. See text for description. (Modified from [61])

characterization of the DNA adduct, and limited specificity, as it is difficult to differentiate the specific types of DNA adducts detected. There is also the possibility of producing artifacts during DNA digestion and sample treatment processes.

1.4.2 Immunoassays

Immunoassays make use of specialized antibodies that recognize and bind to specific carcinogen-DNA adducts. Several different antibodies have been developed that recognize and bind BPDE-DNA adducts. The types of immunoassays used for BPDE-DNA adduct analysis include radioimmunoassays (RIA), fluorescence immunoassays (FIA), chemiluminescence immunoassays (CIA), and enzyme-linked immunosorbent assays (ELISA). In RIA, antibodies labeled with a radioactive isotope bind to the BPDE-DNA adduct. The radioactivity of bound antibody is measured and used to determine the analyte concentration in the sample. FIAs are similar to the RIAs, but the antibody has a fluorescent label rather than a radioactive label. Once the fluorescent label is excited, the fluorescence is measured and can be used to determine the concentration of the analyte. CIAs utilize a chemiluminescent label that is bound to the antibody. The chemiluminescent label produces light when combined with a trigger reagent, and the measured light is used to determine analyte concentration. ELISA makes use of antibodies that are chemically linked to a specific enzyme. Antibodies bind to the analyte of interest, and a substrate for the enzyme is added to the mixture. The product of the reaction between the substrate and enzyme is usually colored or fluorescent, either of which can be measured to determine the amount of antibody binding. These

immunoassays are similar in their use of antibodies to recognize DNA adducts, but differ in their specific detection mechanisms.

Immunoassays are relatively low in cost, easy to use, and applicable to large population studies. The detection limit usually ranges between 1 adduct per 10^8 and 1 adduct per 10^9 nucleotides [59,63,64]. A disadvantage with the immunoassay technique is the possible cross-reactivity of the antibodies. If the chosen antibody is not entirely specific to BPDE-DNA adducts, it may bind to structurally similar compounds and miscalculate the actual amount of BPDE-DNA adducts.

To improve on the sensitivity of DNA adduct analysis, immunoassays may be coupled with analytical instruments. Capillary electrophoresis with laser-induced fluorescence (CE/LIF) can be combined with FIA techniques for highly specific and sensitive adduct measurements [64,65].

1.4.2.1 FIA/CE/LIF

In the fluorescence immunoassay, primary antibodies (1^o-Ab) specific for the BPDE-DNA adduct, fluorescently labeled secondary antibodies (2^o-Ab) that recognize the 1^o antibody, and the DNA sample are mixed together (Figure 1.3). After incubating for a short period of time, three separate fluorescently-labeled products are formed: 2^o-Ab, 2^o-Ab + 1^o-Ab, and the 2^o-Ab + 1^o-Ab + DNA adduct (DNA adduct complex). The

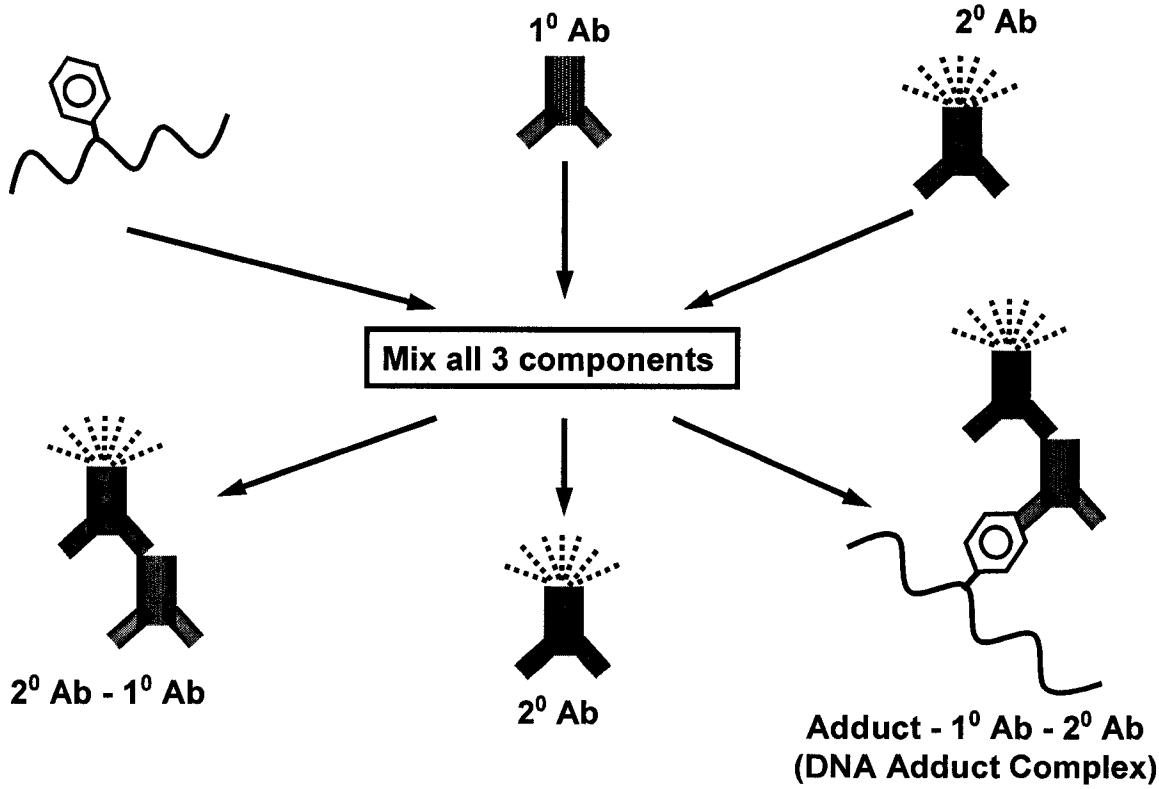


Figure 1.3 Illustration of a fluorescence immunoassay (FIA). The three products shown are the possible configurations of the fluorescently-labeled antibodies. (Modified from [64]).

DNA adduct complex is the product of interest, and can be separated from the free antibodies using CE.

CE is a fast and efficient analytical technique which allows separation of individual molecules based on their size and charge. Movement of molecules through a fused silica capillary (i.d. 20-100 nm) is based on two forces, an electrophoretic force (EPF) and an electroosmotic force (EOF) [66,67]. A high voltage (~20 kV) applied to the capillary creates the EPF; the direction of the force is dependent on the charge of the molecule (Figure 1.4). Buffer movement through the capillary creates the EOF, which drives all molecules towards the negative electrode. Positive ions are driven towards the negative electrode by both the EOF and EPF, resulting in the fastest migration time. Negative ions are driven towards the positive electrode by the EPF, but towards the negative electrode by the EOF, resulting in the slowest migration time through the capillary. Neutral molecules are not affected by the EF, and their movement is guided only by the EOF; this results in a migration time intermediate of the positive and negative ion movement. In the separation of the three fluorescently-labeled products (Figure 1.3), the free antibodies elute first. The DNA adduct complex will elute later due to the negative charge on DNA.

LIF is a highly sensitive and selective detection method, and is compatible with the small sample volumes used in CE [66,68]. In FIA/CE/LIF, the fluorescently-labeled antibodies, either free or bound to DNA adducts, are excited with a helium-neon laser, and the fluorescence is detected. The measured level of fluorescence of bound 2^0-Ab is proportional to the concentration of DNA adducts in the sample.

The combination of immunoassays with CE/LIF is a highly useful method for the detection of DNA adducts. It takes advantage of antibody recognition, CE separation, and the highly sensitive LIF detection. The CE/LIF technique is fast (3-4 min) and requires a small sample size (~50 nL). Past studies have identified a detection limit of approximately 1-3 adducts in 10^9 nucleotides [64,69]. In addition, this assay does not require digestion of the DNA sample, and therefore reduces the possibility of artifacts produced by the DNA treatment procedures, such as with the ^{32}P-postlabelling assay.

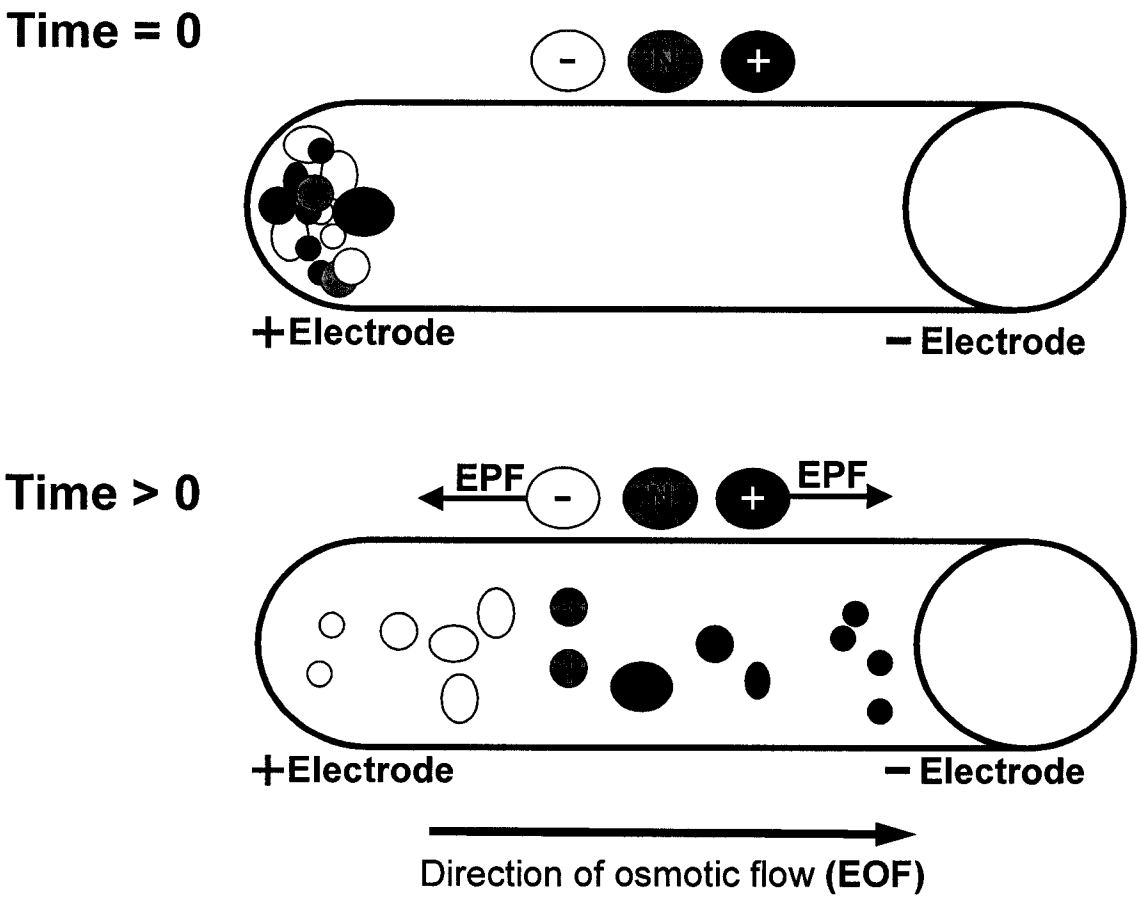


Figure 1.4 Movement of individual molecules through a capillary in CE. EOF: electroosmotic force; EPF: electrophoretic force; (-): negatively-charged molecules; N: neutral molecules; (+): positively-charged molecules.

1.4.3 Mass Spectrometry

Mass spectrometry (MS) coupled with separation techniques (GC, LC, CE) has proven to be a useful method for analyzing DNA adducts. The coupling of these instruments allows the separation and online detection of adducts. MS also enables the determination of structural properties of DNA adducts. Currently, electrospray ionization (ESI) is the most commonly used ionization technique for interfacing LC or CE separations to MS [70]. For LC/ESI/MS, adducts are separated from normal nucleotides using a reversed-phase LC column. As the analytes elute from the column, they pass through a fine needle to an electrospray ionization chamber, where they are converted from small liquid droplets to gas-phase ions. The gas-phase ions are then extracted into the mass spectrometer for analysis.

To identify BPDE-DNA adducts at specific sites within a DNA sequence, labeling of specific DNA bases with a stable isotope (^{15}N) is done prior to LC-ESI-MS/MS analysis of the DNA adducts [71]. As shown in Figure 1.5, ^{15}N-labeled guanine nucleotides are placed at specific positions within DNA oligodeoxynucleotides representing gene sequences of interest. This is followed by treatment with BPDE, enzymatic hydrolysis, and LC-ESI-MS/MS analysis of the nucleoside adducts. BPDE adducts forming at the ^{15}N-labeled guanine are distinct from adducts formed at other sites because of the increased molecular weight of the ^{15}N atom. By varying the site of the labeled nucleotide, it is possible to determine specific sites of BPDE-DNA adduct formation.

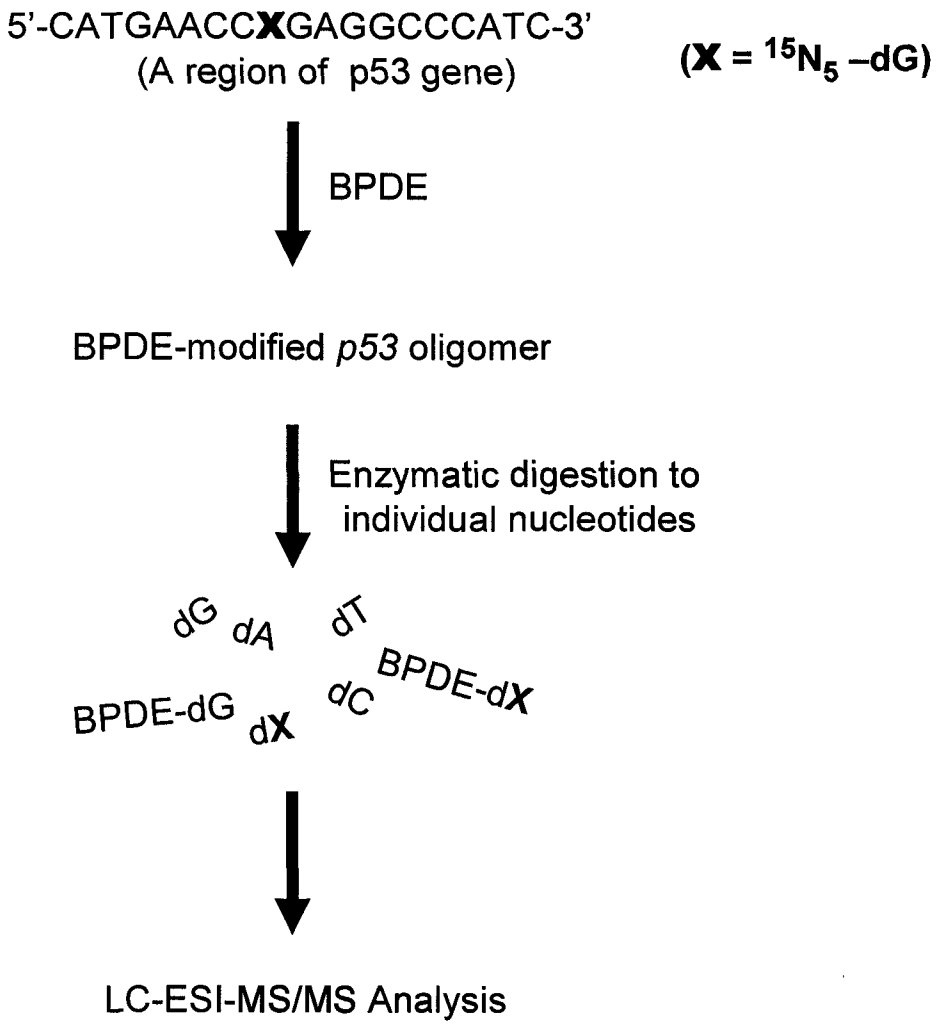


Figure 1.5 Strategy for quantitation of BPDE-deoxyguanosine adducts at specific sites within a DNA sequence [71]. See text for description.

Alternatively, the carcinogen of interest can be isotope labeled and the DNA adducts determined by accelerated MS (AMS) [70]. The carcinogen of interest is labeled with carbon-14 or hydrogen-3 isotopes, and the labeled carcinogen is then administered to cells or animals. DNA is isolated, and extensive procedures are performed to remove any unbound isotope label. The isotope label that remains covalently bound to the DNA is quantified using AMS [72]. AMS demonstrates extremely high sensitivity (1-2 adducts in 10^{12} bases [70]). A disadvantage of AMS is that when assessing individual carcinogen exposure, radioactive labeling is required prior to administration to subjects [72].

More recent techniques have combined LC with nanoelectrospray tandem MS. This assay makes use of enzymatic digestion of DNA, capillary LC separation of individual nucleotides, and internal isotopic standards to quantify adducts, and achieve sensitivities in the range of 40 adducts/10^9 bases [73]. Additionally, because pre-radiolabelling of the adducts is not required, this assay is suitable for *in vivo* applications.

1.4.4 Gene or Sequence Specific Assays

Gene or sequence specific assays allow the identification of the position of DNA adducts. This is important because DNA adducts formed at a biologically significant site of a cancer-related gene are more relevant than those formed in global DNA. As described above, site-specific DNA adduct analysis is based on ^{15}N labeling of the specific nucleotide and LC/ESI/MS/MS. Another gene specific assay is the ligation-

mediated polymerase chain reaction (LMPCR) assay [29,74]. To identify the specific gene in which the BPDE-DNA adduct is formed, purified genomic DNA is first treated with UvrABC nuclease, a DNA nucleotide excision repair enzyme complex isolated from *Escherichia coli*, to cleave at the DNA adduct sites. Next, the DNA fragments are amplified using LMPCR. Specific oligonucleotide primers are used to identify the gene of interest, and the DNA fragments are then separated and visualized by gel electrophoresis. This method is very sensitive because of the PCR amplification. The specific ligation and the UvrABC protein binding to DNA adducts provide the method specificity. A drawback is that that the UvrABC nuclease may not cleave all adducted nucleotides, resulting in potential underestimation of DNA adducts.

1.5 BPDE-DNA Adduct Detection in White Blood Cells

1.5.1 Mononuclear White Blood Cells

Mononuclear white blood cells (MNCs) are a group of white blood cells that do not contain granules in their cytoplasm [75]. MNCs consist of lymphocytes and monocytes, and both cell types play a role in the body's immune system. Lymphocytes make up approximately 20-25% of total white blood cells, and have a long life span (months to years) [76]. Monocytes make up 4-8% of white blood cells, and are short-living (days).

The determination of BPDE-DNA adducts in MNCs offers several advantages. Firstly, blood is an easily obtainable tissue, and this presents a potential use for human biomonitoring. Secondly, through the use of density gradients, MNCs are easy to separate from whole blood. Lastly, multiple studies have identified MNCs as a suitable surrogate tissue for estimating DNA adduct levels in lung cells. For example, Weincke et al. found that DNA adducts in blood MNCs are a suitable predictor for DNA adduct levels in lung cells [77]. Weincke et al. also investigated the effect of smoking status on adduct levels in MNCs and lung tissue [78]. They again concluded that adduct levels in MNCs correlate with adduct levels in lung cells, regardless of individual smoking status. Godschalk et al. also found that BPDE-DNA adduct levels in white blood cells show a correlation with BPDE-DNA adduct levels in the lungs [79]. Additionally, high BPDE-DNA adduct levels in white blood cells have been indicated as a risk factor for lung cancer [50,80].

1.5.2 FIA/CE/LIF for BPDE-DNA Adduct Detection in MNCs

FIA/CE/LIF has been developed as a suitable method for the detection of BPDE-DNA adducts in lung A549 cells [81-83]. To build on this research, we have developed a FIA/CE/LIF technique to detect BPDE-DNA adducts in MNCs.

The ^{32}P-postlabelling assay is the most commonly used assay for BPDE-DNA adduct detection in MNCs. As discussed above (*Section 1.4.1*), there are several

drawbacks to this method, including the potential production of artifacts during DNA digestion, use of radioactive material, and limited specificity for BPDE-DNA adducts.

FIA/CE/LIF helps to overcome these disadvantages. The use of specialized antibodies allows for the specific detection of BPDE-DNA adducts. The production of artifacts is also avoided, as FIA/CE/LIF requires no digestion step. Additionally, FIA/CE/LIF avoids the use of radioactive material. The sensitivity of FIA/CE/LIF is comparable to the sensitivity of other BPDE-DNA adduct detection methods [81]. In addition to these advantages, FIA/CE/LIF is a relatively inexpensive technique.

Two different applications of the developed FIA/CE/LIF method were tested; an *in vitro* assay and an environmental monitoring assessment. These applications are discussed further in *section 1.6* and *section 1.7*.

1.6 FIA/CE/LIF *In vitro* Application: Effect of Glutathione Depletion on BPDE-DNA Adduct Formation

Glutathione-S-transferase (GST) enzymes are thought to be involved in the detoxification of BPDE and BaP [84-87]. Although there are a number of possible BPDE inactivation pathways, including spontaneous hydrolysis and hydrolysis catalyzed by epoxide hydrolase, GST inactivation of BPDE is believed to be the most significant [88]. GST utilizes glutathione (GSH) as a substrate to catalyze conjugation detoxification

reactions. Thus, GSH depletion will ultimately have an effect on the level of GST activity.

Several investigators have observed a relationship between GST phenotype and BPDE-DNA adduct formation in MNCs. For example, Butkiewicz et al. and Guven et al. both found an association between GST null polymorphisms and increased BPDE-DNA adduct formation [85,89]. Furthermore, Wang et al. determined that GST polymorphisms associated with a decrease in GST activity correlate with an increased risk of lung cancer [90]. Additionally, mice pre-exposed to a glutathione depleting agent prior to BaP exposure showed increased tumorogenesis in lungs and stomach compared to mice exposed to BaP only [91]. These findings suggest that GST enzymes are important factors in BPDE detoxification.

MNCs have a considerable amount of GSH [92]. If GST does indeed play a major role in BPDE detoxification in MNCs, co-exposure to BPDE plus a GSH depleting agent would likely result in an increase in the formation of BPDE-DNA adducts. If GST enzymes are found to play a significant part in the BPDE-DNA adduct formation in MNCs, GST enzyme activity and BPDE detoxification should be considered when BPDE-DNA adducts in MNCs are used as biomarkers of exposure.

Evaluating the role of GSH in BPDE-DNA adduct formation allowed us to test the suitability of FIA/CE/LIF for *in vitro* assays. Using FIA/CE/LIF, we observed significant increases in levels of BPDE-DNA adducts in MNCs co-exposed to BPDE and

a GSH inhibitor compared to MNCs exposed to BPDE alone. *In vitro* studies provide a valuable approach to improving our understanding of the mechanisms of BaP carcinogenesis. As will be discussed further in Chapter 3, we have shown that FIA/CE/LIF is a suitable method for MNC *in vitro* analyses. Further *in vitro* studies utilizing this method may be used to help clarify the role of DNA adducts in BaP-induced carcinogenesis.

1.7 FIA/CE/LIF Biomonitoring Application: Detection of BPDE-DNA Adducts in Smokers

There have been inconsistent results regarding the association between BPDE-DNA adducts in MNCs and exposure to cigarette smoke. Several studies have shown significant correlations between adduct levels and smoking [78,93,94], though it is not entirely clear which measures of smoke exposure would be predictive of adduct levels in target tissues [95]. Among different studies, BPDE-DNA adducts have been related to cigarette tar content [5], pack-years [96], daily cigarette consumption [79], or age of smoking initiation [78]. Wiencke et al. determined that adducts in MNCs are a suitable surrogate for adducts in lung cells, a primary target tissue for BPDE [77,78]. However, it is not known to what extent MNC adduct levels are related to tobacco smoke exposure.

A number of studies have indicated there is no relationship between smoking and MNC adducts [51,97-99]. For example, van Maanen et al. found no differences in MNC adduct levels between smokers and nonsmokers [100]. Also, Rojas et al. did not

observe any differences between adducts in smokers compared to nonsmokers; however, they did see an enhancing effect of smoking on adduct levels in coke oven workers [101].

Because of the lack of consistency between the above-mentioned studies, the relationship between adducts and smoking requires further investigation. Chapter 4 describes in detail the use of FIA/CE/LIF to detect BPDE-DNA adducts in MNCs. Though the detected adducts were not quantifiable, additional modifications to the method may help to improve sensitivity. It is hoped that FIA/CE/LIF DNA adduct detection will eventually provide a sensitive and selective method to better measure the presence of BPDE-DNA adducts in MNCs.

1.8 Thesis Objectives

This project was comprised of three objectives:

i. to develop and optimize a FIA/CE/LIF method for the detection of BPDE-DNA adducts in MNCs;
ii. to utilize FIA/CE/LIF for *in vitro* analysis and assess the role of glutathione in BPDE-DNA adduct formation;
iii. to test the ability of the FIA/CE/LIF method to detect BPDE-DNA adducts in MNCs of smokers.

The development and optimization of the FIA/CE/LIF method is thoroughly discussed in Chapter 2. The optimized method was then used to assess the dose-response relationship between cellular BPDE exposure and BPDE-DNA adduct formation. To evaluate the usefulness of FIA/CE/LIF, the technique was used in an *in vitro* study and in blood testing of individuals exposed to BaP. Chapter 3 examines the role of glutathione in BPDE-DNA adduct formation through *in vitro* exposure to BPDE and a glutathione depleting agent. Chapter 4 assesses the ability of the developed method to detect BPDE-DNA adducts in smokers.

1.9 References

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Chapter 2
Development of FIA/CE/LIF for the Detection of BPDE-DNA Adducts in Mononuclear White Blood Cells

2.1 Introduction

Our laboratory previously developed a FIA/CE/LIF technique suitable for the detection of BPDE-DNA adducts in lung cells [1,2]. This assay offers high specificity to BPDE-DNA adducts and is an extremely sensitive detection method. We extended this work by developing a FIA/CE/LIF method to measure BPDE-DNA adducts in MNCs. As discussed in Chapter 1, there are several BPDE-DNA adduct techniques available, including ^{32}P-postlabeling, immunoassays, mass spectrometry, and sequence specific assays. ^{32}P-postlabelling is the most commonly used BPDE-DNA adduct analysis technique. However this assay is not always ideal; disadvantages include the potential production of artifacts during DNA digestion, use of radioactive material, and limited specificity for BPDE-DNA adducts [3,4]. FIA/CE/LIF offers several advantages over the ^{32}P-postlabeling assay. Primarily, the use of specialized antibodies allows specific detection of BPDE-DNA adducts. Also, because FIA/CE/LIF does not require DNA digestion, the production of artifacts is avoided. Additionally, FIA/CE/LIF avoids the use of radioactive material. The sensitivity of FIA/CE/LIF is comparable to the sensitivity of other BPDE-DNA adduct detection methods [1,5].

A number of factors need to be optimized for efficient FIA/CE/LIF analysis of MNC DNA adducts. Parameters such as antibody concentrations, antibody incubation

time, and use of stability proteins will all have an effect on the binding of antibodies to the BPDE-DNA adduct complex. CE separation factors, including buffer pH, electric field strength, and sample injection time, will affect the peak resolution, elution time, and signal intensity of CE analysis. In the present study, these factors were assessed to determine the most optimal conditions for the method.

The use of MNCs for BPDE-DNA adduct analysis offers several advantages. A number of studies have identified MNCs as a suitable surrogate tissue for estimating DNA adduct levels in target tissue [6-8]. BPDE-DNA adducts in white blood cells have also been indicated as a risk factor for lung cancer [9,10]. Furthermore, blood is a relatively easy tissue to obtain and offers the potential for human biomonitoring. Development of a specific and sensitive method for BPDE-DNA adduct analysis in MNCs may help to clarify the relationship between BaP exposure and adduct formation, as well as characterize the role of BPDE-DNA adducts in BaP induced carcinogenesis. This may potentially lead to the use of BPDE-DNA adducts as biomarkers of exposure and preclinical markers of effect.

2.2 Experimental Methods

2.2.1 Development of BPDE-DNA Adduct Detection Method

2.2.1.1 BPDE preparation

(±)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). A stock solution of 5 mM BPDE in dimethylsulfoxide (DMSO, Sigma, Oakville, ON) was prepared on the day of blood collection, and frozen at -20^{0}C until required for incubation (~4 hours). BPDE solutions were thawed at room temperature immediately prior to use.

2.2.1.2 Mononuclear Cell Isolation

Ethical approval for this study was given by the Health Research Ethics Board at the University of Alberta. Consent was obtained from all blood donors who participated in the study. Volunteer blood samples (~40 mL) were drawn into BD Vacutainer® blood collection tubes containing 0.081 mL of 15% EDTA (Preanalytical Solutions, Franklin Lakes, NJ), and processed immediately after collection. Two different MNC isolation methods were used, both of which utilized Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) density gradients. Method 1 followed the manufacturer's suggested protocol [11]. Whole blood was layered on top of room temperature Ficoll-Paque Plus and centrifuged. The MNC layer was pipetted out and the isolated MNCs were washed twice with a salt solution. Method 2 was a slightly altered

version of the manufacturer's suggested protocol. Whole blood was centrifuged, followed by collection of the buffy coat layer. The buffy coat was then layered on top of room temperature Ficoll-Paque Plus and centrifuged. The MNC layer was pipetted out and the isolated MNCs were washed twice with RPMI 1640 medium (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS, Sigma, Oakville, ON), and 1% penicillin/ streptomycin (Sigma, Oakville, ON). For both methods, cells were suspended in the RPMI medium, and cell viability was assessed using trypan blue staining followed by hemacytometer counting. Leukocyte counts in whole blood and isolated MNCs were determined using a Beckman Coulter A^c-T Series Analyzer (Miami, FL).

2.2.1.3 BPDE Incubation

RPMI medium (5mL) was added to 6 cm polystyrene culture plates and warmed to 37^0C. BPDE (or DMSO control) was added to each plate according to final BPDE concentration required (1.0, 0.5, and 0 μM). Final DMSO concentrations did not exceed 0.1% (v/v). An appropriate amount of cell suspension was added to each plate for a total concentration of 10^6 cells/mL. Cells were incubated at 37^0C for 2 hours, followed by two washings with phosphate buffered saline (PBS, Sigma, Oakville, ON). BPDE can be hydrolyzed in aqueous solutions, and a short incubation of 2 hours is optimum to achieve BPDE-DNA adduct formation and minimize the hydrolysis of BPDE.

2.2.1.4 DNA Isolation

Three different DNA isolation methods were attempted in this study, including DNAzol (Invitrogen, Burlington, ON), Trevigen Genomic DNA Isolation Kit (Trevigen, Gaithersburg, MD), and BioRad Aquapure Genomic DNA Isolation Kit (BioRad,

Hercules, CA). DNA was isolated following the suggested protocol from the respective manufacturer [12-14]. Briefly, DNAzol utilizes a guanidine-detergent lysing solution to disrupt the cells and hydrolyze RNA. DNA is then selectively precipitated with the addition of 100% ethanol (Commercial Alcohols Inc, Brampton, ON), air-dried, and dissolved in ddH_2O. The Trevigen kit utilizes a non-phenol, chloroform-based biphasic extraction to isolate DNA. Following the biphasic extraction, the DNA is precipitated with isopropanol (Fischer Scientific, Ottawa, ON), air-dried, and dissolved in ddH_2O. The BioRad kit works by first lysing the cells with an anionic detergent in the presence of a DNA stabilizer. The sample is treated with an RNA-digesting enzyme to remove contaminating RNA, followed by a salt precipitation to remove protein and other contaminants. DNA is then precipitated with isopropanol, air-dried, and dissolved in a DNA hydration solution. DNA concentration was measured at A_{260} (SmartSpec 3000™, BioRad, Hercules, CA) using ddH_20 as a blank. Samples were stored in water or DNA hydration solution at 4^0C until analysis.

2.2.1.5 FIA/CE/LIF

Anti-benzo(a)pyrene-guanosine monoclonal antibody (clone 8E11) (1^0-Ab), of ~150 kDa in size, was purchased from Trevigen (Gaithersburg, MD). The Zenon Alexa Fluor 546 Mouse IgG1 Fab fragment (2^0-Ab), of ~50 kDa in size, was purchased from Molecular Probes (Eugene, OR). 1x Tris-Glycine (TG) buffer (25mM Tris and 192 mM glycine at pH 8.3) was prepared from a 10x stock solution (BioRad, Hercules, CA) using ddH_2O. Buffer pH was adjusted using glacial acetic acid (Fischer, Fair Lawn, NJ) or Tris

powder (Invitrogen, Carlsbad, CA). The stabilizing agents bovine serum albumin (BSA) and human immunoglobulin G (IgG) were both purchased from Sigma (Oakville, ON).

Previous studies have shown that single-stranded DNA, rather than double-stranded DNA, is optimal for FIA/CE/LIF analysis [15]. Thus, DNA in 1x-TG buffer (pH 7.5) was heated at 95^0C for 15 min to allow DNA to denature. Immediately following denaturation, DNA was chilled on ice for a minimum of 30 min, and remained chilled until analysis. The antibody labeling solution was prepared using varying molar ratios of the 2^0:1^0 antibodies (6:1, 9:2, 3:1, 3:2, and 1:1), followed by addition of varying concentrations of BSA (0, 100, 500, and 1000 μg/ml) or human IgG (0, 50, and 100 μg/mL). A small volume of the antibody labeling solution was mixed with the denatured DNA, and incubated in the dark for various lengths of time (5-30 min). When initially starting the optimization process, the following conditions, derived from previous experiments, were used: 50 μg/mL DNA, 1000 μg/mL BSA, 20 μg/mL 1^0-Ab, 20 μg/mL 2^0-Ab, and an incubation time of 30 min.

The capillary electrophoresis – laser induced fluorescence instrument was laboratory built and has previously been described in detail [1,2,15-17]. Briefly, a CZE 1000R power supply (Spellman, Plainview, NY) provided voltages in the range of 0 to 30kV (Figure 3.1). A green helium-neon laser (λ = 543.5 nm, output 5mW) (Melles Griot, Carlsbad, CA) was focused onto a window in the capillary wall (6 cm from the end of the capillary) for on-column detection. Fluorescence was collected at 90^0 using a 60X microscope objective, filtered with a 580DF40 band-pass filter, and detected with a

Novatron photomultiplier tube (PMT). The sampling rate for collection of data was 10 Hz. The LabVIEW program (National Instruments, Austin, TX) on a Macintosh IIci computer was used to control sample injection time/voltage, separation voltage, and run time, as well as collection of data from the PMT. Fused silica capillaries (20 μm i.d., 148 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ). The instrument was aligned using the fluorescent dye tetramethylrhodamine (TMR, Sigma, Oakville, ON).

Samples were electrokinetically injected into the capillary by applying a voltage of 10 kV for a brief period of time (5 or 10s). The capillary was washed every two samples with 100 mM NaOH for 5 min, 20 mM NaOH for 5 min, ddH_2O for 5 min, and 1x-TG buffer (pH 8.3) for 3 min. The following conditions, derived from previous experiments, were used when initially starting the optimization process: 484 V/cm (15 kV running voltage, 31 cm capillary), 10 s injection time at 10 kV, and 1x TG running buffer (pH 8.3). The sample injection time (5-10 seconds) and the electric field (323, 484, 517, and 645 V/cm) were varied to determine the optimum electrophoretic conditions. The sample and running buffers (1x TG, pH 7.5 and 8.3, respectively) were kept consistent with previous experiments [1,2,15-17].

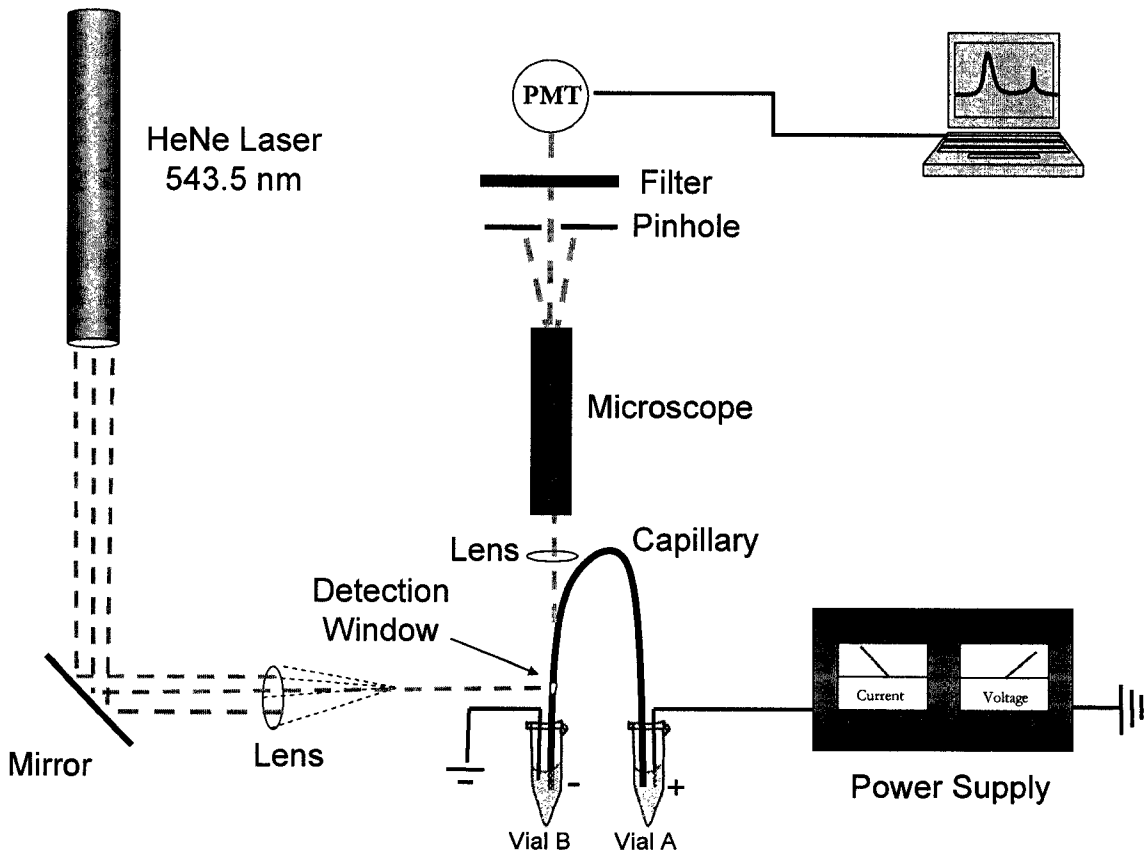

Figure 2.1 Schematic of the CE-LIF laboratory-built instrument. The sample is injected at the positive end of the capillary (Vial A), and the negative end of the capillary sits in a buffer reservoir (Vial B). PMT: photomultiplier tube.

2.2.1.6 CE/LIF Data Analysis

Peak area and peak height are two methods used for peak quantitation, the former being more commonly used. Although peak area is sensitive to integration start and end times, it is more accurate for quantifying asymmetric peaks and is the main approach for identifying relationships between peaks and analyte concentration [18]. Previously developed FIA/CE/LIF methods also utilized peak area for quantitation [2,15,16]. The relative peak area (RPA), which indicates the proportion of fluorescent antibody bound to DNA adducts, was used as the measurement of comparison in this study. RPA was determined by comparing the area of the adduct complex peak to total peak area. Standard error (SE) was used as the measurement of variability for RPAs. Generation of electropherograms and integration of peaks was performed using Igor Pro Version 4.0 (Wavemetrics, Lake Oswega, OR).

2.2.2 BPDE Dose-Response Assessment

2.2.2.1 BPDE preparation

A stock solution of 5 mM BPDE dissolved in DMSO was prepared on the same day as the experiment. The 5 mM solution was further diluted into solutions of 0.6 mM and 0.15 mM. All three solutions were frozen at -20^{0}C until required for incubation (~4 hours). BPDE solutions were thawed at room temperature immediately prior to use.

2.2.2.2 Mononuclear Cell Isolation and BPDE Incubation

MNCs were isolated from a 70mL blood sample as described above (*Section 2.2.1.2, method 2)*. 70 mL of whole blood provided a sufficient number of MNCs for 18 incubation culture plates. Six different BPDE concentrations (0, 0.0625, 0.125, 0.25, 0.50, and 1.00 μM) were assessed in triplicate. The cell concentration was 10^6 cells/mL in 3 mL of RPMI 1640 medium. Cells were incubated at 37^0C for 2 hours, followed by two washings with PBS. Trypan blue and hemacytometer counting were used to assess cell viability before and after BPDE incubations.

2.2.2.3 DNA Isolation

DNA was isolated using the BioRad Aquapure Genomic DNA Isolation Kit (*Section 2.2.1.4*). DNA was dissolved in a DNA hydration solution, and DNA concentration was measured at A_{260}. All samples were diluted to 100 μg/mL with DNA hydration solution, and stored at 4^0C until analysis.

2.2.2.4 FIA-CE-LIF

FIA-CE-LIF is described in detail above (*Section 2.2.1.5*). The following immunoassay conditions were used for DNA adduct detection: 50 μg/mL DNA, 1x TG sample buffer (pH 7.5), 50 μg/mL human IgG, 10 μg/mL 1^0-Ab, 20 μg/mL 2^0-Ab, and 15 min incubation time. The electrophoretic conditions used were 1x TG running buffer (pH

8.3), a 29 cm capillary with the detection window at 23cm, and a 10s injection time at 10 kV. Electrophoresis was run at 15 kV (517 V/cm) for 4 min.

2.2.2.5 CE/LIF Data Analysis

Adduct complex RPAs were calculated and compared between samples. Weighted least squares regression analysis (SPSS 15.0, Chicago, IL) was used to assess the relationship between dose and response. Statistical significance of the linear regression line was evaluated with the student's *t*-test.

2.3 Results and Discussion

2.3.1 Development and Optimization of BPDE-DNA Adduct Detection Method

2.3.1.1 MNC Isolation

Initial attempts to isolate MNCs utilized the Ficoll-Paque Plus suggested protocol. However, recoveries were very low, ranging from 1- 2.5%. GE Healthcare, the manufacturer of Ficoll-Paque Plus, states that MNC recoveries with this product should range from 40-60% [11]. It is possible that the majority of the MNCs became trapped in aggregates of red blood cells (RBCs) and migrated to the bottom layer of the tube. To overcome this problem, we attempted to remove the bulk of the RBCs prior to addition of Ficoll-Paque Plus. In this method, the buffy coat, rather than whole blood, was applied to

Ficoll-Paque density gradients. Following these alterations, MNC recoveries were 33-52%, suggesting that MNC aggregation in RBCs was likely a reason for the low MNC recovery. All further experimentation utilized the adjusted method to isolate MNCs.

Following every MNC isolation procedure (n=7), cell viability was assessed using trypan blue staining followed by hemacytometer counting. Cell viabilities were consistently high, ranging from 97-99%. Cell viability was also assessed post-incubation once during the method development process; following 2 hours of exposure to 1 μM BPDE or DMSO control, cell viabilities were 98% and 95%, respectively.

2.3.1.2 DNA Isolation

DNAzol was the first DNA extraction reagent used for DNA isolation. The DNAzol suggested protocol for isolation of DNA in cell suspensions was followed, however three major problems were encountered during the procedure. Firstly, the DNAzol protocol indicated that cell lysis should occur immediately after mixing of cells with DNAzol, however the cells had to be left in DNAzol overnight in order to be fully lyzed. Secondly, the protocol stated that DNA precipitation should occur within 1-3 min of addition of ice cold ethanol, yet the samples required freezing at -20^{0}C for an extended period of time (1+ hours or overnight) for DNA precipitation to occur. Thirdly, there were difficulties dissolving the DNA in water. Incubating the DNA samples at 37^{0}C overnight, and addition of 8mM NaOH were both attempted in order to improve dissolution, but neither procedure helped. Expected DNA yields were calculated based

on a theoretical value of 7.1 μg DNA per 10^6 cells [12]. The manufacturer states normal yields should range from 70-100% [12], however DNA recoveries ranged from 1-16%, with an average value of 10%.

Although several studies report successful use of DNAzol for DNA extraction from MNCs [19-21], DNAzol was not an acceptable extraction reagent for this project. Possible reasons for the observed problems may be that the DNAzol was outdated, the protocol was not followed properly, and/or the ratio of MNCs to DNAzol volume was not appropriate.

Because problems were experienced with DNAzol, DNA extraction kits from Trevigen and BioRad were tried. Isolation of DNA using the Trevigen DNA Extraction Kit resulted in DNA that was slow to precipitate and a low recovery (~14%). The BioRad DNA Extraction Kit was the easiest and most efficient method for DNA extraction. There were fewer problems with cell lysis and DNA precipitation, as both steps occurred more quickly than with the other two kits. Additionally, dissolution of DNA occurred relatively quick in comparison to dissolution of DNA extracted using DNAzol. DNA recoveries with the BioRad kit ranged from 26-96%, with an average value of 43%. Based on the percent recovery and ease of procedure, the BioRad kit provided the most efficient DNA isolation method and was used for all further experiments in this study. DNA recoveries in subsequent experiments consistently ranged between 30-50%.

2.3.1.3 Immunoelectrophoresis Method Optimization

The first condition tested was antibody concentration. To ensure all DNA adducts are bound, it is important that the concentration of the antibodies be in excess of the adduct concentration [5]. Additionally, a major factor for determining optimal antibody concentrations is the 2^0:1^0 molar ratio, as the mass of the 1^0-Ab is ~3 times that of the 2^0-Ab [22]. In the present study, the antibody molar ratio had a significant effect on adduct complex peak signal (Table 2.1). At a 1:1 ratio, adduct complex RPAs were very small. A ratio of 3:2 resulted in low total peak area, indicating there was not enough 2^0-Ab to produce a sufficient signal. Antibody ratios of 6:1(a) and 9:2 had very high total peak areas, indicating the amount of 2^0-Ab was too high. The 3:1 and 6:1(b) ratios provided reasonable total peak areas and acceptable adduct complex RPAs. However, the 6:1(b) ratio had adduct complex RPAs double to those of the 3:1 ratio of antibodies, indicating improved complex formation. The 6:1(b) ratio was tested repeatedly to assess consistency of results. Average RPAs and standard errors are displayed in Table 2.2. The manufacturer's suggested molar ratio is 3:1, however, they also state that higher ratios may be needed in some experiments [22]. This supports our finding of an optimal antibody molar ratio of 6:1.

Table 2.1 The Effect of Varying Antibody Molar Ratios on RPAs and Total Peak Areas in BPDE-DNA Adduct Analysis of MNCs.

	Label Kit 1	Label Kit 2	Label Kit 3	Label Kit 4	Label Kit 5	Label Kit 6
[1^0 Ab] (μg/mL)	60	20	20	20	20	10
[2^0 Ab] (μg/mL)	20	10	40	30	20	20
Antibody Molar Ratio (2^0:1^0)	1:1	3:2	6:1(a)	9:2	3:1	6:1(b)
Avg RPA (%) (0.5 μM BPDE)	1.5	5.8	6.1	5.5	5.8	12.6
Avg RPA (%) (1.0 μM BPDE)	3.6	9.1	7.4	12.9	9.3	21.9
Total Peak Area (Arb Units)	516	272	744	790	557	573

Table 2.2 Average RPAs and Standard Errors (SE) from BPDE-DNA Adduct Analysis of MNCs Using a 2^0:1^0 Antibody Ratio of 6:1 (Label Kit 6).

[BPDE] (μM)	Avg RPA (%)	SE (%)	n
1.0	21	1.2	4
0.5	11	1.2	4
0	0.5	0.1	2

n=number of repeat FIA/CE/LIF analyses

Stability proteins have been shown to enhance and stabilize antibody-antigen interactions during FIA/CE/LIF analysis [2]. This effect is thought to be due to interactions of non-specific protein with antibodies and DNA, which may stabilize the conformations of the antibodies and adducts for optimal binding. The effect of the stability proteins BSA and human IgG on adduct detection were assessed in this study. DNA originated from MNCs exposed to 0.5 μM BPDE for 2 hours, and the concentration of the stability proteins was varied during BPDE-DNA adduct analysis. As shown in Table 2.3, when BSA concentration was 1000 μg/mL, adduct complex RPA was lowest (12%). At BSA concentrations of 500 μg/mL and 100 μg/mL, adduct complex RPA improved to 20 ± 0.2% and 22 ± 2.4%, respectively. Similar RPAs were found when using 100 μg/mL IgG (24.5 ± 6.3%) or no stability protein (25.2 ± 3.5%). The highest adduct complex RPA was found with 50 μg/mL IgG (37.0 ± 0.6%). Additionally, IgG

appeared to be a better stability agent than BSA as sharper peaks were produced and there was less peak splitting. The combination of 50 μg/mL IgG with 90 μg/mL BSA was also evaluated, however the addition of BSA made no apparent improvement on complex stability. For all further experiments, 50 μg/mL IgG was used as the stability protein. Previous studies also determined IgG to be a better stabilizing agent than BSA [2].

Table 2.3 The Effect of BSA and IgG on BPDE-DNA Adduct Complex RPAs.

Stability Protein	Concentration (μg/mL)	Avg RPA (%)	SE (%)	n
BSA	1000	12	-	1
BSA	500	20	0.2	2
BSA	100	22	2.4	2
IgG	100	25	6.3	2
IgG	50	37	0.6	2
None	0	25	3.5	2

n=number of repeat FIA/CE/LIF analyses

Antibody and adduct incubation times ranging from 5 min to 30 min have been reported previously [1,2,16,17]. In this study, incubation time did not appear to cause any major changes in adduct migration time or peak shape, as shown in Figure 2.2. However, an incubation time of 5 min produced a slightly broader adduct complex peak. The effect of antibody incubation time on RPA is displayed in Table 2.4. RPAs from

DNA exposed to 0.25 μM BPDE did not differ significantly with varying antibody incubation times; however, slight variations were observed with DNA exposed to 0.5 μM BPDE. A possible explanation for this observation is that there is more variation in FIA/CE/LIF analysis with higher DNA adduct concentration. Alternatively, antibody incubation time may have a greater effect on adduct binding when adduct concentrations are higher. Because of this possibility, the optimal antibody incubation time was identified using DNA from the 0.50 μM BPDE plate. At an incubation time of 25 min, the relative adduct complex peak area was low, though experimental error cannot be excluded as a cause. Incubation times of 10, 15, or 20 min allowed for maximum adduct complex formation, while remaining time efficient. All further experiments utilized an incubation time of 15 min as this produced the highest adduct complex RPA, and thus was an optimal time for antibody-adduct complex formation.

Table 2.4 Effect of Antibody Incubation Time on RPA from FIA/CE/LIF Analysis of MNCs Exposed to 0.25 μM or 0.50 μM BPDE.

Incubation Time	Relative Peak Area (%)	
	0.25 μM BPDE	0.50 μM BPDE
30 min	7.7	36.9
25 min	8.7	21.9
20 min	8.7	36.3
15 min	8.5	41.6
10 min	8.9	35.0
5 min	9.0	32.6

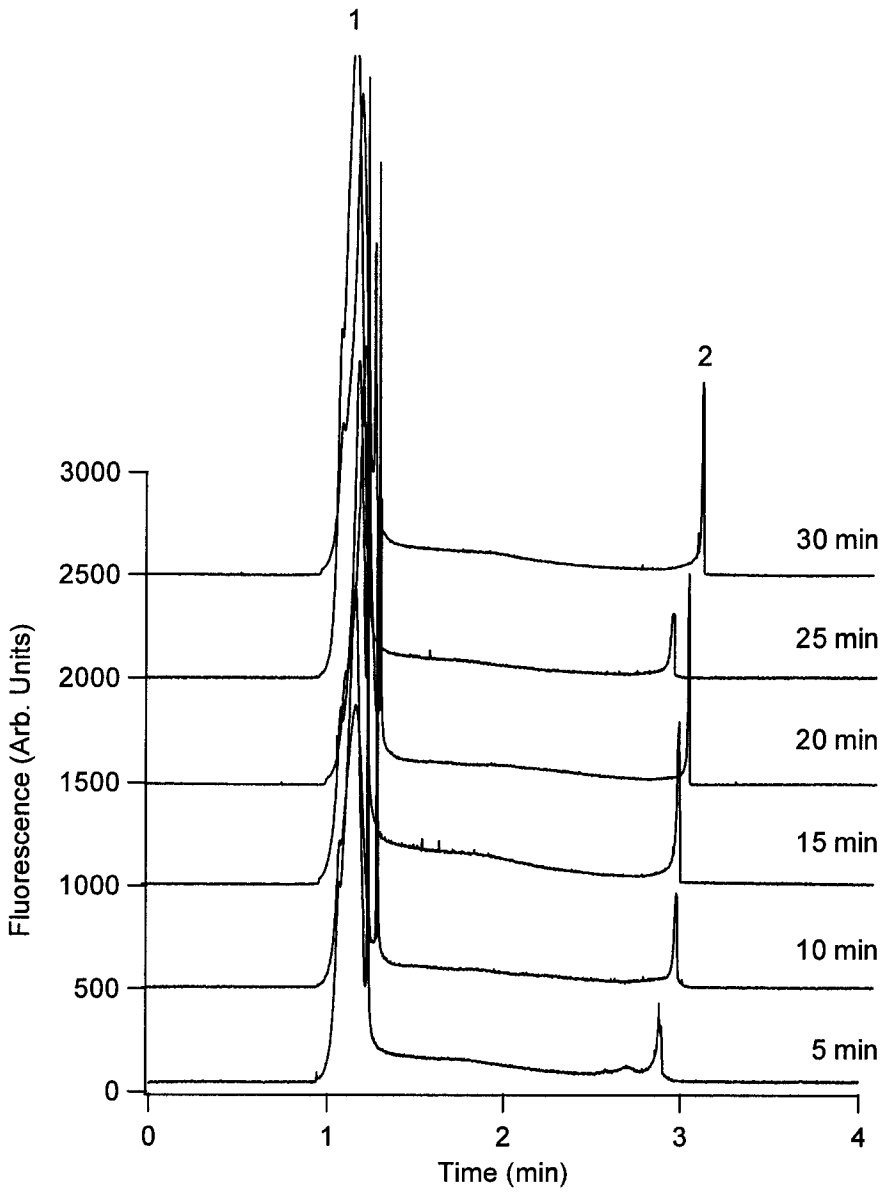

Figure 2.2 Effect of antibody incubation time on adduct complex formation. DNA samples from MNCs exposed to 0.5 μM BPDE were incubated with 1^0 and 2^0 antibodies for 5 to 30 min. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes; RPA: relative peak area.

The electric field had a significant effect on peak migration time. Increasing the electric field will increase the EOF, which results in faster movement of molecules through the capillary [5]. Separation was assessed using a 31 cm capillary with the voltage ranging from 10 to 20 kV. The adduct complex peak elution time for electric fields of 645, 484, and 323 V/cm was on average 2.2, 3.1, and 4.7 min, respectively (Figure 2.3). Adduct complex RPA remained relatively the same at all three voltages. Resolution of peaks occurred at the highest voltage, however there was some instrument static produced with the use of 20 kV. The peak elution time was then assessed using a shorter capillary (29 cm) with 15 kV separation voltage. This produced an electric field of 517 V/cm, which allowed for faster separation (~3 min) than at 484 V/cm, but without the production of instrument static. An electric field of 517 V/cm was used for all remaining experiments.

A sample injection time of 5 seconds and 10 seconds were both assessed. A shorter injection time of 5 seconds did not have an effect on peak shape. As expected, the total peak area was smaller with an injection time of 5 seconds compared to 10 seconds, because a smaller amount of fluorescently labeled antibody was injected into the capillary. A sample injection time of 10 seconds was chosen for all further experiments as this produced the optimal signal intensity.

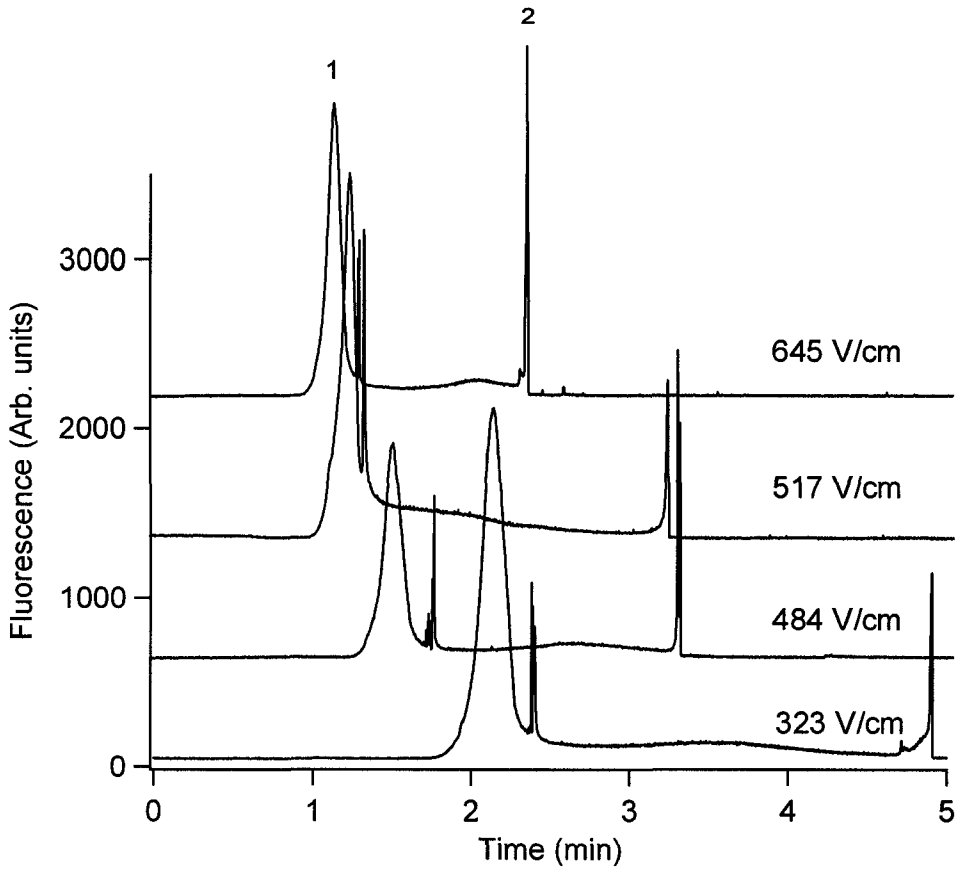

Figure 2.3 Effect of electric field strength on CE separation. DNA samples from MNCs exposed to 0.5 μM BPDE for 2 hours were separated at different electric field strengths. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

2.3.1.4 CE/LIF Data Analysis

In carrying out quantitative analysis of the peaks, two problems were encountered. Firstly, the signal did not always return to baseline after the free antibody peak. Secondly, there was tailing of the free antibody peak. For these reasons, the two peaks of interest overlapped, making deconvolution techniques necessary for peak integration.

The perpendicular drop method of peak integration is among the most reliable and simple of deconvolution techniques [23]. Though there are inherent inaccuracies in this type of integration, the perpendicular drop is widely used in peak area determination in chromatography. The perpendicular drop is most accurate when the overlapped peaks are similar in size and width. However, when peaks are different in size and width, the peak area of the smaller peak becomes overestimated while the peak area of the larger peak becomes underestimated [23,24]. When peak widths are significantly different or when peaks have considerable tailing, Gaussian skimming can be a more reliable approach [24-26]. This deconvolution technique takes into account the Gaussian shape of the parent peak, which in our case is the free antibody peak.

In Figure 2.4, the electropherogram from free antibodies is overlaid with a typical sample electropherogram. To help prevent possible overestimation of peak 2, the Gaussian skimming method of integration was performed to account for the tailing of peak 1. Because our data processing software did not have capabilities to automatically

perform Gaussian skimming, a slightly modified version of this integration was done manually (Figure 2.5a). The percentage of tailing at the end of peak 1 was consistently in the range of 4-5% of the total area of peak 1. For example, the shaded area in Figure 2.5a accounts for 4.8% of peak 1. To account for this tailing in the peak 1/peak 2 overlap and minimize the overestimation of peak 2 RPA, 4.8 % of the area of peak 1 (11 (arbitrary units)) was subtracted from the area of peak 2 and added to the area of peak 1 (Figure 2.5b). The adjusted peak areas were then used to calculate RPA. In this example, the original RPA of peak 2 was 54%, while the adjusted RPA of peak 2 was 51%. For all further experiments, antibodies were analyzed without DNA on each day of analysis to determine the percentage of tailing for that particular day; typical tailing percentages ranged from 4.2 – 4.8%.

The separation point between the two peaks was chosen as the point at which the adduct complex peak appears to deviate from the free antibody peak (Figure 2.4). Although somewhat arbitrarily chosen, separating the peaks at this data point allowed quantification of the entire peak area for peak 2. The elevated baseline observed at the start of peak 2 may be due to dissociation of bound antibodies from DNA adducts [27,28]. This is supported by the observation that a higher BPDE exposure concentration resulted in a higher baseline level between peak 1 and peak 2. This is understandable because the formation and dissociation of the antibody-adduct complex are in equilibrium; the proportion of dissociation stays the same with varying DNA adduct levels. This means that at higher adduct concentrations, the absolute number of dissociating antibodies will be higher than at lower adduct concentrations, resulting in a

higher baseline following peak 1. The antibodies have a certain binding affinity for the antigen, and this binding affinity can change with pH, temperature, or solvent [29]. The conditions inside the capillary may have favored some dissociation of the DNA adduct complex, leading to the elevated baseline. Additionally, the Ab manufacturer states that some loss of Ab may occur over time, as the Ab is not covalently bound to the antigen [22].

As errors in any deconvolution method are difficult to avoid, the reproducibility of the quantitative analysis becomes a very important concern [30]. The manual Gaussian skim data analysis technique provided the most consistent results for peak area quantification (see *Section 2.3.2* for discussion on variability). The tailing of the free antibody peak was accounted for, as well as the apparent dissociation of the antibodies from the adduct complex. All further data was analyzed using this method.

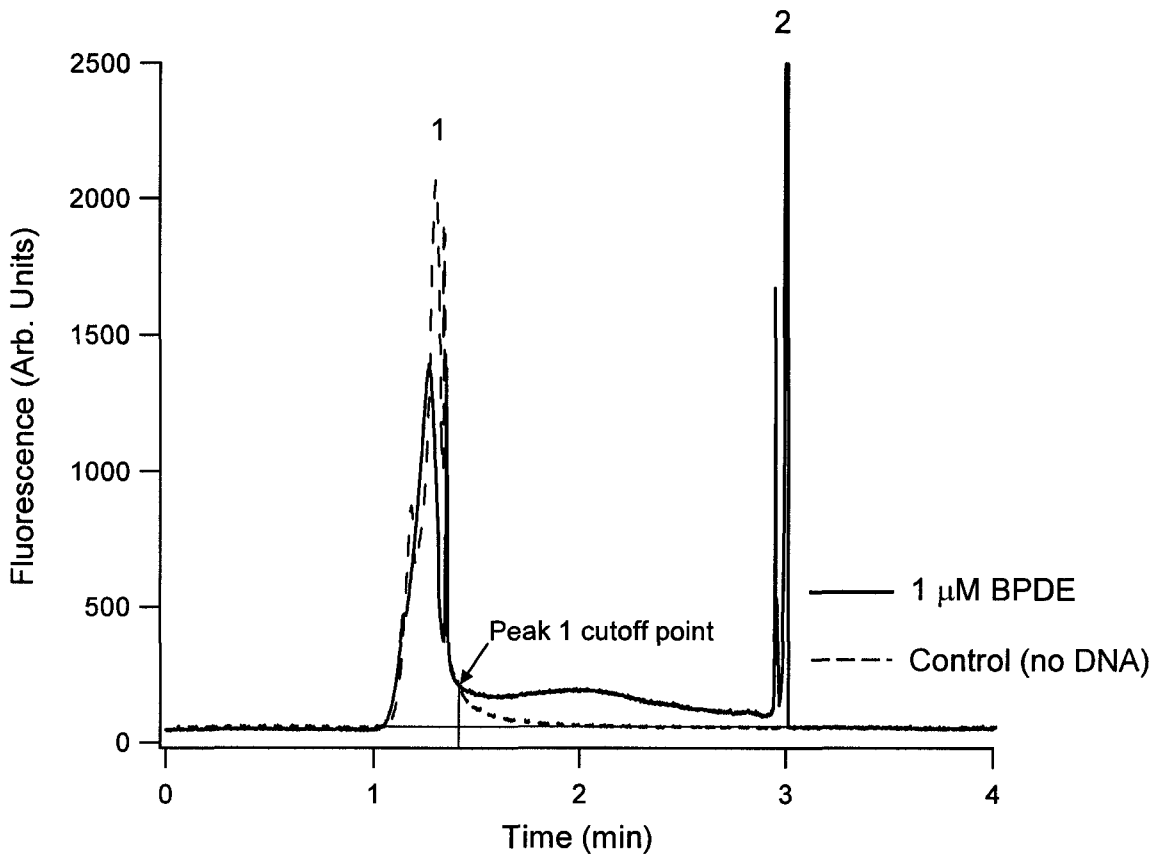

Figure 2.4 Overlay of the control electropherogram (2^0 and 1^0antibodies only, no DNA) and a typical 1.0 μM BPDE sample electropherogram. Peak 1 cutoff point is indicated at the point where the two electropherograms begin to deviate. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

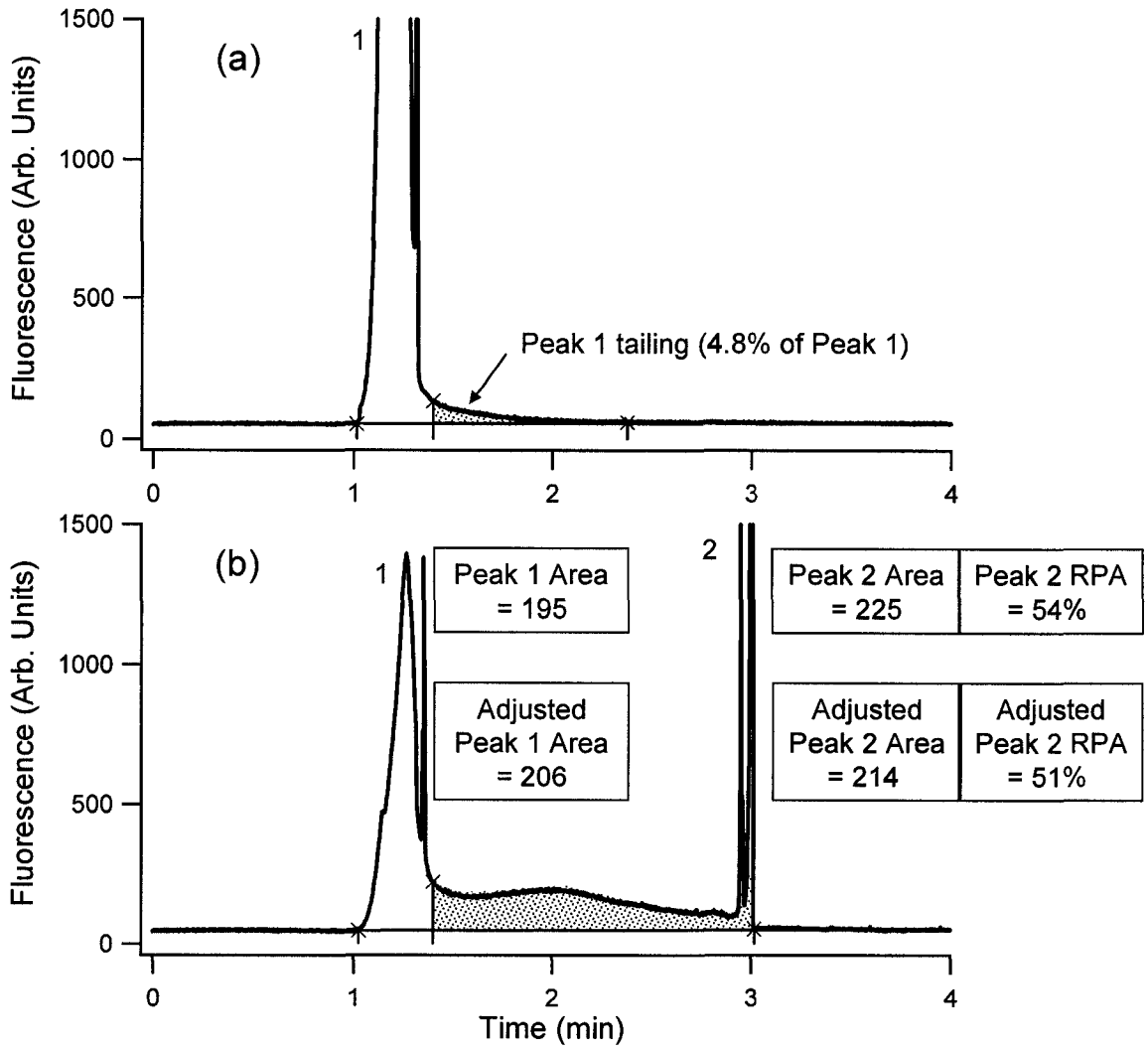

Figure 2.5 (a) BPDE-DNA adduct analysis of 2^0 and 1^0 antibodies only (no DNA). Shaded area indicates peak 1 tailing portion. (b) BPDE-DNA adduct analysis following cellular exposure to 1.0 μM BPDE. Adjusted peak 2 values account for 4.8% tailing of peak 1. See text for description of calculations. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

2.3.2 BPDE Dose-Response Assessment

Figure 2.6 displays typical electropherograms from BPDE-DNA adduct analysis following cellular exposure to varying concentrations of BPDE. As BPDE concentration increased, the size of the BPDE-DNA adduct complex peak (peak 2) also increased. RPAs for all samples were quantified and plotted against concentration to determine if a dose-response relationship exists. Figure 2.7 displays data from the dose-response analysis, and a linear correlation was observed. Because the standard error was not consistent across all of the data, weighted least squares regression, which accounts for unequal variance, was used to analyze the regression. Utilizing weights that are inversely proportional to the variance yields more precise parameter estimates [31]. The weighted least squares linear equation was $y = 43x + 2.1$. The t-test of H_0: slope = 0 vs H_A: slope > 0 gives a *p*-value of <0.0005, indicating there is a significant correlation between BPDE concentration and RPA.

Within sample variability and between sample variability were both assessed in this study (Table 2.5). Within-sample variability refers to the variability in RPA values from a minimum of three analyses using DNA from a single incubation plate. The variability appeared to increase with increasing BPDE concentration. A possible reason for this may be because as the level of adducts increases, there may be more variation in the level of antibody-adduct binding during each immunoassay. This variation may become more apparent at higher adduct levels, and thereby increase the standard error. Another source of error may be in the data analysis technique. The end point of peak 1 is

somewhat subjective, and the identification of this endpoint became more difficult to determine at higher BPDE concentrations. This may have lead to slight variations in calculated RPA values. However, moving the endpoint of peak 1 up to 25 data points in either direction had a minimal impact on calculated RPAs.

Table 2.5 Average RPA and Standard Errors (SE) from BPDE-DNA Adduct Analysis of MNCs Exposed to Various Concentrations of BPDE

[BPDE] (μM)	Avg RPA (%)	Avg Within-Sample SE (%)	Between-Sample SE(%)	n
1.00	46.8	1.9	4.6	3
0.50	28.5	1.7	6.5	3
0.25	16.3	1.4	2.9	3
0.13	10.8	0.9	2.2	3
0.06	5.9	0.8	3.0	3
0	2.3	0.7	0.2	3

n=number of separate incubation plates

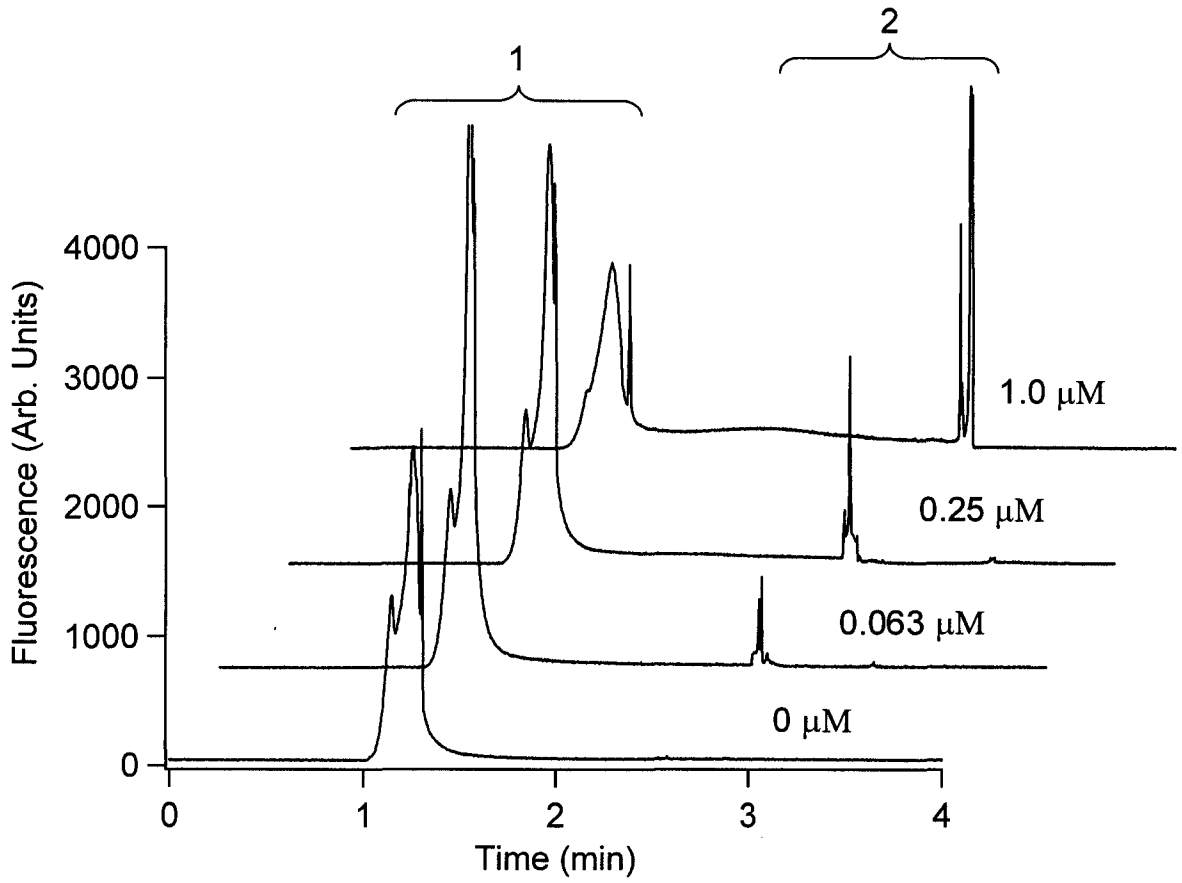

Figure 2.6 Electropherograms from BPDE-DNA adduct analysis following cellular exposure to varying concentrations of BPDE. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

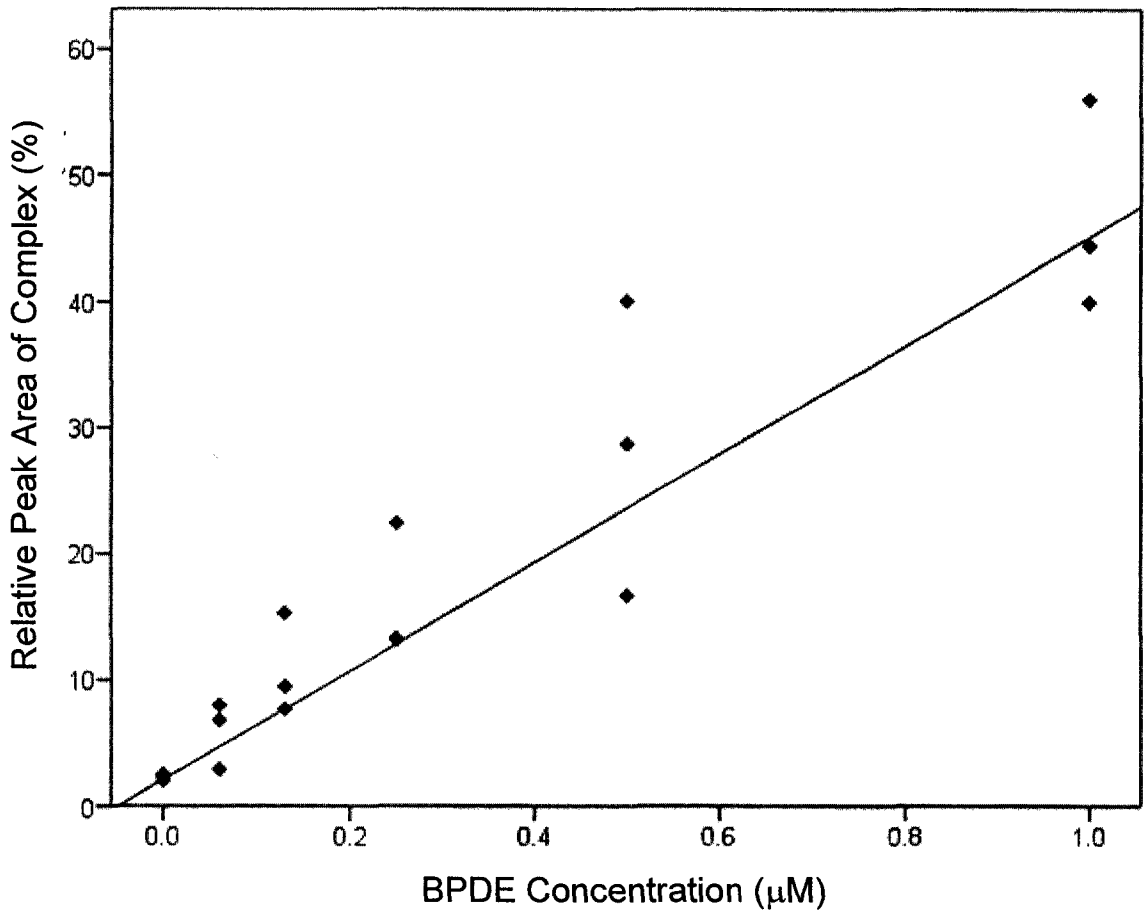

Figure 2.7 Dose-response relationship between BPDE concentration and DNA adduct complex relative peak areas. Isolated MNCs were incubated with varying concentrations of BPDE for 2 hours at 37^0C. Incubations were performed in triplicate for each concentration. Best fit line was determined using weighted least squares regression.

Between sample variability refers to the variability in average RPA values from analyses using DNA from 3 separate incubation plates exposed to the same BPDE concentration. As indicated in Table 2.5, between sample variation was found to be higher than within sample variation at all BPDE concentrations except for the control (0 μM BPDE). It is expected that the between sample variability would be greater than within sample variability as there are more potential sources of error between multiple plates than for a single plate. For example, it was difficult to ensure an accurate and equal dispersion of cells in each culture plate due to clumping of MNCs. Therefore, the cell concentration may have varied from plate to plate, resulting in varied formation of adducts between plates. Another possibility may be slight variations in pipette volumes, perhaps leading to differences in BPDE concentrations in each plate.

At this time, it is not known to what extent the variability can be attributed to sampling variation rather than analytical variation. The within sample variability and the between sample variability can be better estimated with the use of a known BPDE-DNA adduct standard for FIA/CE/LIF analysis. Alternatively, analysis of the same MNC samples using an alternative acceptable method would allow comparisons of the variation in FIA/CE/LIF to the variation observed with other techniques.

2.4 Conclusion

This study demonstrates development of a new assay for the detection of BPDE-DNA adducts in MNCs. Following optimization of several different assay

parameters, we were able to detect BPDE-DNA adducts in MNCs exposed to nanomolar concentrations of BPDE. Additionally, we observed a linear correlation between BPDE concentration and adduct complex formation. Although only relative comparisons were made in this study, quantitative analyses of adduct formation are possible with the use of BPDE-DNA adduct standards. This would also allow the determination of DNA adduct detection limits. Applications of this method in human biomonitoring studies and *in vitro* assays will further test the suitability of FIA/CE/LIF for BPDE-DNA adduct analysis in MNCs.

2.5 References

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Chapter 3
Effect of Glutathione Depletion on BPDE-DNA Adduct Formation

3.1 Introduction

Possible BPDE detoxification pathways in the body include spontaneous hydrolysis, hydrolysis catalyzed by epoxide hydrolase, and glutathione (GSH) inactivation [1,2]. Of the three, the latter is considered the most significant [3,4]. As shown in Figure 3.1, glutathione-S-transferase (GST) enzymes catalyze the addition of GSH to electrophilic regions of BPDE [5,6]. The added GSH group inactivates BPDE, as well as increases its hydrophilicity. This in turn allows the body to eliminate BPDE more easily.

Buthionine sulfoximine (BSO) is a potent inhibitor of GSH synthesis [7]. BSO binds and inhibits γ-glutamylcysteine synthetase (γ-GCS), an enzyme involved in the rate-limiting step of GSH biosynthesis [8,9]. The inhibition of γ-GCS, and thus GSH synthesis, eventually leads to the cellular depletion of GSH. The resultant decrease in GSH causes a reduction in GST enzyme activity. BSO has been successfully used for cellular GSH depletion in a number of *in vitro* and *in vivo* studies [10-15]. Since GST enzymes are considered to play an important role in BPDE detoxification, a decrease in GSH, and thus a loss of GST enzyme activity, should theoretically result in an increase in BPDE-DNA adduct formation.

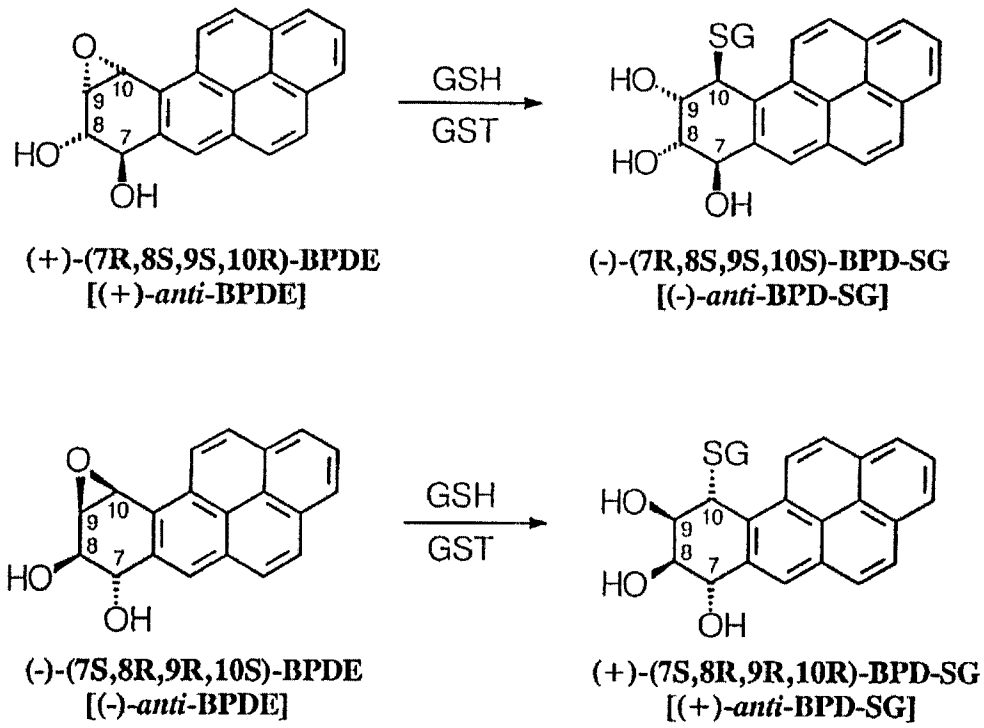

Figure 3.1 Structures of BPDE enantiomers and their resultant GSH conjugates [5].

Correlations between GST phenotypes and BPDE-DNA adducts in MNCs have been assessed in multiple studies [16-19]. Additionally, *in vivo* assays have demonstrated that exposure to BSO prior to BaP exposure increases BaP-induced tumorogenicity [14]. However, *in vitro* assays evaluating the effect of GSH and GST enzyme activity specifically on BPDE-DNA adduct formation in MNCs have not been done. To test whether GSH and GST enzymes play an important role in BPDE detoxification in MNCs, cells were co-exposed to BPDE plus BSO. The levels of BPDE-DNA adducts in these cells were then compared to adduct levels in MNCs exposed to BPDE only. In carrying out this study, we were also able to verify the use of FIA/CE/LIF for *in vitro* DNA adduct analyses in MNCs.

3.2 Experimental Methods

A stock solution of 5 mM BPDE dissolved in DMSO was prepared on the same day as the experiment, and frozen at -20^{0}C until required for incubation. The BPDE solution was thawed at room temperature prior to use. A 1 mM stock solution of DL-buthionine-[S,R]-sulfoximine (Sigma, Oakville, ON) in RPMI was also prepared in the morning prior to the experiment. The BSO solution was stored at 4^{0}C until required for incubation.

MNCs isolated from a 40 mL blood sample (as described in Chapter 2) were exposed to either 150 μM BSO alone, 0.5 μM BPDE alone, or 150 μM BSO + 0.5 μM BPDE. The cell concentration was 10^6 cells/mL in 3 mL of RPMI 1640 medium. Four

different exposure protocols for the plates containing both BSO and BPDE were evaluated: *i.* co-exposure with BSO and BPDE for 2 hours, *ii.* 2 hours pre-exposure with BSO followed by 2 hours BPDE exposure, *iii.* 4 hours pre-exposure with BSO followed by 2 hours BPDE exposure, and *iv.* 14 hours pre-exposure with BSO followed by 2 hours BPDE exposure. Pre-treatment with BSO for 2 hours (exposure protocol *ii*) was chosen as the optimal exposure time. This experiment was repeated using MNCs from a second donor, utilizing three separate incubation plates for each of the BSO control group, the BPDE control group, and the BPDE+BSO group. Trypan blue staining and hemacytometer counting were used to assess cell viability before and after BSO and BPDE incubations.

DNA isolation was performed using BioRad Aquapure Genomic DNA Isolation Kit. The immunoassay and electrophoretic conditions for BPDE-DNA adduct detection, as well as the data analysis procedures, were the same as described in Chapter 2. Statistical differences between adduct complex RPAs following exposure to BSO, BPDE, or BSO + BPDE were analyzed with the student's *t*-test.

3.3 Results

Following MNC exposure to BSO and BPDE simultaneously for 2 hours, there was a slight, although not significant, difference seen in RPA; BPDE control RPA was 11.4 ± 0.3% compared to 13.1 ± 0.9% for co-exposure to BPDE + BSO (Table 3.1). Pre-exposure of MNCs to BSO for 2 hours or 4 hours prior to addition of BPDE (19.4 ± 0.8%

and 19.8 ± 2.4%, respectively) resulted in a higher RPA than cells exposed only to BPDE. However, only the 2 hour pre-incubation was statistically significant when compared to MNCs exposed to BPDE only; the high variance observed in the 4 hour pre-incubation plates was the reason the RPA value lacked statistical significance. When MNCs were pre-exposed to BSO for 14 hours prior to addition of BPDE, RPA (12.0 ± 0.7%) returned to levels similar to the BPDE control group. Cell viability was not affected following exposure to 150 μM BSO for 14 hours or BPDE for 2 hours, indicating this dose of BSO was not fatal to the cells (Table 3.2).

Table 3.1 Average RPAs and Standard Errors (SE) from BPDE-DNA Adduct Analysis of MNCs Exposed to 150 μM BSO and 0.5 μM BPDE

Pre-exposure time with BSO (hr)	Avg RPA (%)	SE (%)	n
no BSO	11.4	0.3	3
0 (coexposure)	13.1	0.9	3
2	19.4*	0.8	3
4	19.8	2.4	3
14	12.0	0.7	3

*statistically significant compared to 'no BSO' in student's t-test ($p<0.05$);
n=number of repeat FIA/CE/LIF analyses

Table 3.2 Cell Viabilities Following Exposure to 150 μM BSO and/or 0.5 μM BPDE

Exposure Time		Cell Viability	
BSO	BPDE	pre-exposure	post-exposure
2 h	2 h	98%	97%
4 h	2 h	98%	95%
4 h	-	98%	98%
14 h	-	98%	98%

Pre-treatment with BSO for 2 hours was chosen as the optimal exposure time for BPDE-DNA adduct formation. Figure 3.2 displays typical electropherograms for MNCs exposed to BSO, BPDE, and BSO + BPDE. Peak 2 (BPDE-DNA adduct complex peak) was not present in cells exposed to BSO only. Peak 2 from cells exposed to BSO + BPDE was found to be approximately double that of cells exposed only to BPDE. RPA values for cells exposed to BSO, BPDE, or BSO + BPDE were 1.1 ± 0.5%, 11.4 ± 0.3%, and 23.3 ± 1.2%, respectively. These RPA values represent the average RPA from three separate incubation plates, and are in good agreement with the results from the initial experiment (Table 3.1). The differences in RPA values between the three groups were all statistically significant ($p<0.05$).

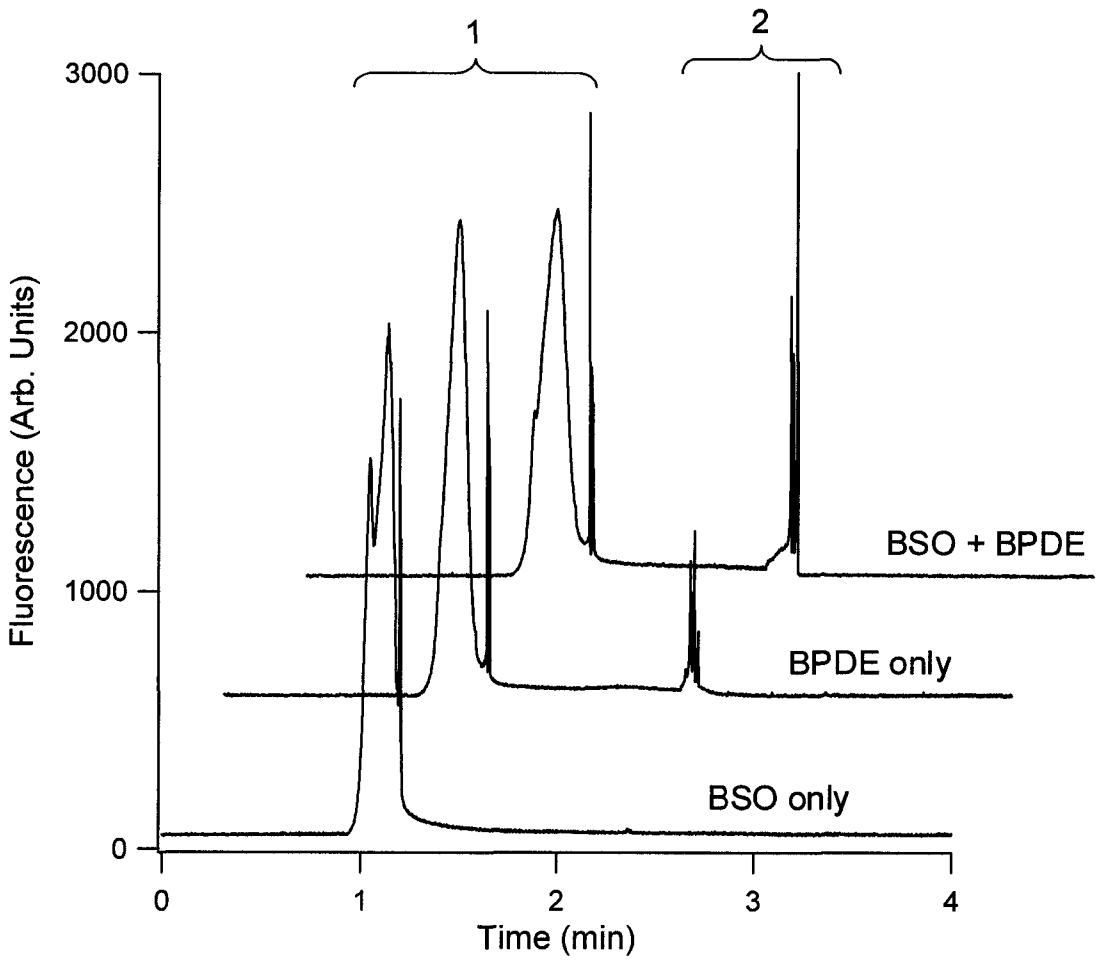

Figure 3.2 Sample electropherograms from FIA/CE/LIF adduct analysis following cellular exposure to 150 μM BSO only, 0.5 μM BPDE only, or 150 μM BSO + 0.5 μM BPDE (two hours apart). Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

3.4 Discussion

BSO, a selective inhibitor of γ-GCS, is the most widely used GSH depleting agent [8]. Following inhibition of γ-GCS, cells continue to use GSH for normal cellular processes; this eventually depletes cellular stores of GSH [8]. The rate of GSH depletion depends on the rate at which GSH is used, and this varies from tissue to tissue.

In the present study, the optimal pre-incubation time was both 2 hours and 4 hours, although the 4 hour pre-incubation lacked statistical significance. Pre-incubation times of 0 hours and 14 hours did not alter the formation of BPDE-DNA adducts compared to controls. Because BSO requires time to deplete GSH [8], it is expected that 0 hours of pre-incubation would not have a major effect on GSH levels. However, it is not clearly understood why BPDE-DNA adduct levels in the 14 hour BSO incubation plates were similar to control levels. Other studies have shown that BSO causes GSH depletion in MNCs over a range of exposure times and concentrations, varying from 2-72 hours and 10-1000 μM, respectively [22-25]. Based on these results, GSH levels should still be depleted after 14 hours of exposure to 150 μM BSO.

BSO binds very tightly, though not covalently, to γ-GCS [8]. BSO has an initial binding constant of ~100 μM (for rat γ-GCS), and is considered an irreversible γ-GCS inhibitor [20]. However, over time, BSO may dissociate from γ-GCS, allowing γ-GCS to once again catalyze GSH formation. Although the dissociation rate is extremely slow (1-3% per hour), even a small amount of available γ-GCS can rapidly replete GSH when

initial GSH levels are low [8]. Alternatively, BSO may have induced transcription of genes encoding γ-GCS, as has been observed previously [21,22]. This may have generated sufficient γ-GCS to replenish GSH after 14 hours of BSO exposure. Lee et al. found significant variations in the rates of GSH depletion and GSH recovery among different rat tissues following exposure to BSO [23]. GSH regeneration was fastest in the liver and slowest in the heart, with GSH nadirs ranging from 5 hours to 24 hours. Therefore, the possibility exists that GSH levels had been repleted after 14 hours. However, because we did not measure cellular GSH levels in parallel with the DNA adduct analysis, we are not certain whether the adduct level observed in the 14 hour incubation plate was due to a repletion of GSH or as a result of other cellular processes.

Another possible explanation for the lower adduct level observed in the 14 hour plate, compared to the 2 or 4 hour BSO plates, may be due an induction of alternative BPDE detoxification pathways. For example, the detoxification of BPDE catalyzed by epoxide hydrolase may have been induced in the 14 hour plates but not the 2 or 4 hour plates. A previous study has shown that in the absence of glutathione, there was an increase in the level of epoxide hydrolase-derived metabolic products of the carcinogen styrene oxide, indicating induction of epoxide hydrolase when GST detoxification is minimized [24]. If a loss of cellular GSH for an extended period of time (ie. 14 hrs) allows for the induction of epoxide hydrolase, then this offers an alternative explanation for the effects observed in our study. Experiments assessing epoxide hydrolase activity following exposure to BSO and BPDE would allow us to confirm or rule out this possibility.

We found that a depletion of GSH in MNCs resulted in an increase in BPDE-DNA adduct formation. Because BSO reduces GST activity [25], the increase in adduct formation is likely the result of a decrease in BPDE detoxification caused by a loss of GST enzyme activity. It is possible that the depletion of GSH affected other cellular processes such as BPDE efflux or uptake, however cellular uptake of PAHs is likely mediated by lipoproteins, albumin, and/or fatty acids [26,27]. Alternatively, GSH may react spontaneously with electrophiles, however these reactions most often require catalysis by GST enzymes [8]. The increased BPDE-DNA adduct formation observed in this study is likely attributed to a loss of GST activity. In future work, measures of GST activity following BSO exposure may help to support this conclusion. Our finding in this study is consistent with other results suggesting that GST polymorphisms associated with a decrease in GST activity correlate with increased BPDE-DNA adduct formation [28-30]. Because GST enzymes play an important role in the formation of BPDE-DNA adducts, it is important that GST enzyme activity and BPDE detoxification be considered when BPDE-DNA adducts in MNCs are used as biomarkers of exposure.

3.5 Conclusion

In the present study, the role of GSH in BPDE detoxification in MNCs was assessed. When MNCs were exposed to a GSH depleting agent prior to BPDE exposure, the formation of BPDE-DNA adducts increased significantly compared to BPDE exposure alone. This suggests that GSH, and possibly GST enzymes, play an important role in the detoxification of BPDE in MNCs. Thus, GST activity and BPDE

detoxification should be taken into consideration when MNC BPDE-DNA adducts are used as biomarkers of exposure.

Using FIA/CE/LIF as a detection method for BPDE-DNA adduct analysis, we observed significant differences in adduct formation between cells exposed to various contaminants. Thus, this study verified FIA/CE/LIF as a suitable method for *in vitro* analysis of BPDE-DNA adducts. *In vitro* studies utilizing this method may help to further characterize BPDE-DNA adducts, and improve our understanding of the mechanisms of BaP carcinogenesis.

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Chapter 4
Detection of BPDE-DNA Adducts in MNCs of Smokers

4.1 Introduction

The use of DNA adducts as a measure of carcinogen exposure provides a more realistic approach to exposure assessments than ambient concentrations, as DNA adducts identify the amount of substance that has interacted with DNA. Many studies have sought to identify the relationship between smoking and BPDE-DNA adduct formation, in hopes of potentially using BPDE-DNA adducts as biomarkers of cigarette exposure [1-5]. However, it is not clear which measures of smoke exposure, such as pack-years, number of daily cigarettes, or cigarette tar content, best relate adducts to smoking [6]. The lack of consistency in this area of research suggests that the relationship between smoking and BPDE-DNA adducts requires further investigation.

The majority of the above-mentioned studies utilized the ^{32}P-postlabelling assay for adduct detection. Perhaps the inconsistencies lie in the method of detection method, as possible introduction of artifacts and/or lack of specificity may have confounded the results. FIA/CE/LIF is a highly specific method that avoids the introduction of artifacts; use of this method for adduct analysis may potentially provide a better estimate of actual BPDE-DNA adduct levels in MNCs of exposed individuals. Improved DNA adduct detection may help to clarify the relationship between smoking and adduct formation.

In the present experiment, we obtained blood from smokers and performed BPDE-DNA adduct analysis. The study was preliminary in nature; only two smokers were tested and smoking status was the only personal information provided. Nevertheless, we were able to test the suitability of FIA/CE/LIF as a potential method for human biomonitoring studies.

4.2 Experimental Methods

One tube (6-7 mL) of whole blood was drawn from each of 2 smokers and 1 non-smoker. The investigator was blinded to smoker status throughout the entire analysis. Isolation of MNCs and extraction of DNA were performed immediately upon collection of blood. Detailed procedures have been previously described in Chapter 2.

All immunoassay and electrophoretic conditions remained the same as described in Chapter 2, with the exception of DNA concentration. The DNA concentration was increased from 50 to 150 μg/mL in attempts to improve detection.

4.3 Results and Discussion

Figure 4.1 displays electropherograms from two smokers and one non-smoker. The BPDE-DNA adduct complex peak (peak 2) is present in both smokers, while this peak is not seen in the non-smoker. This indicates the presence of BPDE-DNA adducts in MNCs of smokers. However, in both smokers, peak 2 is very small. The RPA values

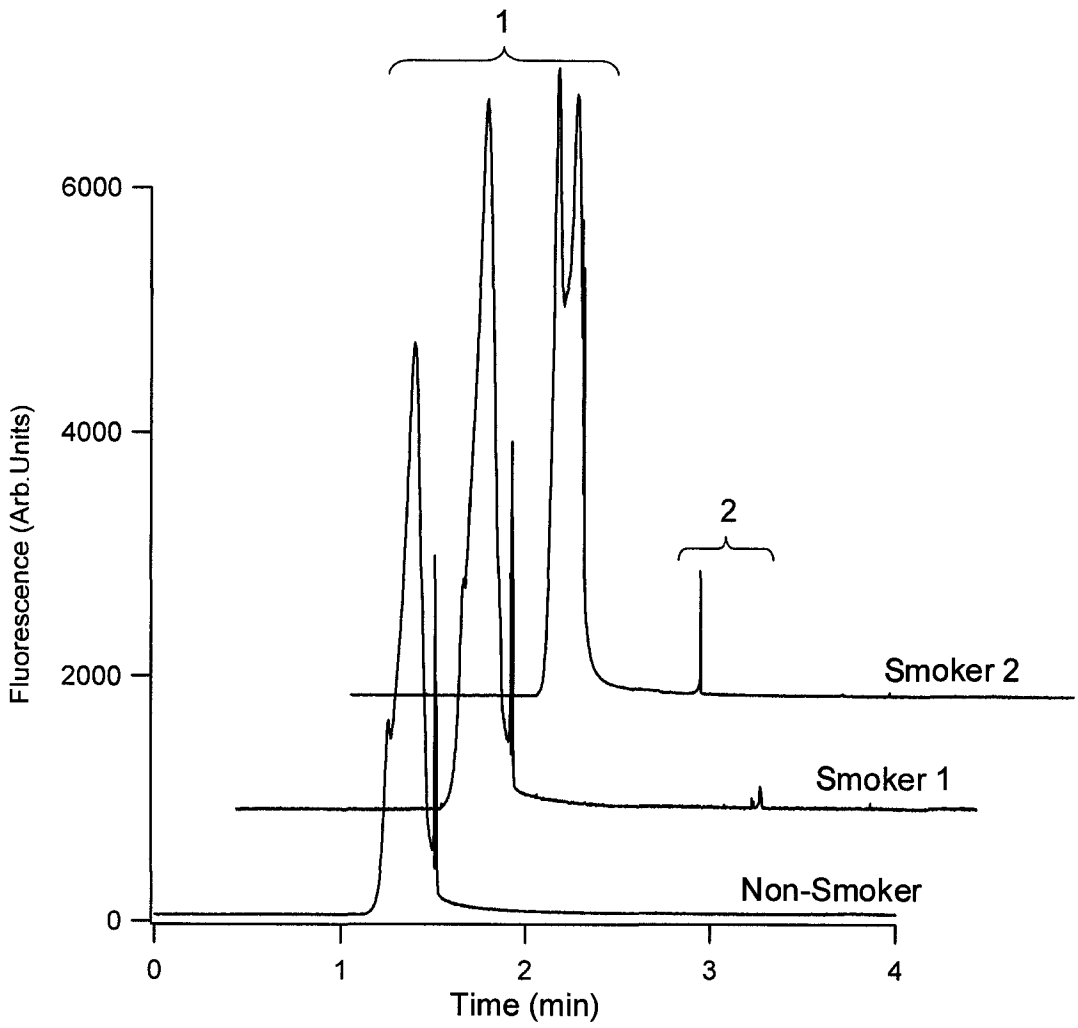

Figure 4.1 Electropherograms from BPDE-DNA adduct analysis of MNCs from 2 smokers and 1 non-smoker. Peak 1: free 2^0 and 2^0-1^0 antibody complexes; Peak 2: 2^0-1^0-BPDE-DNA complexes.

were not found to be significantly different for the smokers compared to the non-smoker (0.0, 1.6, and 0.9% for smoker 1, smoker 2, and non-smoker, respectively). Thus, peak 2 is considered detectable but not quantifiable. A DNA concentration of 150 μg/mL, rather than 50 μg/mL, from smoker 1 was analyzed to see if increasing the DNA concentration would improve detection. However, the signal did not increase. The RPA of the BPDE-DNA adduct complex peak using 150 μg/mL DNA was 1.5%, which was similar to the RPA using 50 μg/mL of DNA (1.6%). This suggests a possibility that the level of BPDE-DNA adducts in smoker 1 was very low.

Another point to note is that the BPDE-DNA adduct complex retention time was different for the two smokers. Peak 2 in smoker 1 elutes at 2.8 min, while peak 2 in smoker 2 is seen at about 2 min. A possible reason for this difference could be because the blood from the two smokers was processed and analyzed on different days. There may have been more DNA fragmentation in smoker 2 during the DNA isolation process, resulting in smaller DNA that eluted more quickly. Alternatively, differences in buffer pH, capillary conditioning, and/or capillary cleanliness could also be the source of varying retention times. However, regardless of this discrepancy, peak 2 in both smokers can still be attributed to the BPDE-DNA adduct complex, as it is normal for retention time to vary slightly between runs.

As the BPDE-DNA adduct complex peak was small in both smokers, further improvements to this method are necessary. Perhaps a concentration of DNA higher than 150 μg/mL will be required, either by drawing more blood from donors or finding a DNA

isolation method with higher extraction efficiency. Another way to improve sensitivity may be to use a sheath flow cuvette for off-column detection rather than on-column detection. The column-sample interface in on-column detection produces light scattering, thereby increasing background; this is avoided with the use of a sheath flow cuvette as there is no light scattering at the sample-sheath interface [7]. Sheath flow cuvettes can provide concentration detection limits up to four orders of magnitude better than on-column fluorescence detectors [7-9].

It is also possible that these two smokers simply had very low levels of adducts. Because we did not include asking any personal information of the donors in our ethical approval, we could not determine how much these individuals smoked, how long they have been smoking for, when they last smoked, or the brand name of their cigarettes. DNA adduct analyses of individuals known to be heavy smokers may clarify whether the low level of detected DNA adducts is due to the method or due to low adduct levels in the smoker.

Analysis of a standard with a known concentration of BPDE-DNA adducts would allow the determination of method sensitivity. Comparing results of BPDE-DNA adduct analysis of the same MNC samples to other acceptable adduct analysis methods may also help to verify suitability of FIA/CE/LIF. Future work in this area will allow us to identify whether FIA/CE/LIF is an appropriate method for human DNA adduct biomonitoring.

4.4 Conclusion

The goal for this portion of the project was to see if it was possible to detect BPDE-DNA adducts in MNCs of smokers using FIA-CE-LIF. We were able to observe the BPDE-DNA adduct complex peak in smokers, however the peak was not quantifiable. Although the lack of quantifiable adducts may be due to low levels of BPDE-DNA adducts in the donors' blood, it is likely that the sensitivity of the method will still need to be improved. These improvements for detection are necessary if this method is to be used for BPDE-DNA adduct detection in humans exposed to BaP.

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Chapter 5
Conclusions and Future Work

This project involved the development of a FIA/CE/LIF method for BPDE-DNA adduct analysis in MNCs. The advantages of FIA/CE/LIF over other currently used adduct detection techniques include high specificity, fast analysis time, no digestion of DNA, and no use of radioactive material. The optimized FIA/CE/LIF method was used in two different applications; firstly, an *in vitro* assay, and secondly, a human biomonitoring study. The *in vitro* study utilizing FIA/CE/LIF for MNC adduct analysis was successful, as we were able to confirm that GST and GSH are important in the detoxification of BPDE in MNCs. The use of FIA/CE/LIF for human biomonitoring experiments requires further research, as BPDE-DNA adducts in MNCs of smokers were detectable but not quantifiable.

Use of FIA/CE/LIF in future *in vitro* studies may further assess the role of GST enzymes in BPDE detoxification. Specific reagents can be used to block individual GST isozymes to evaluate which forms of GST are most significant in BPDE detoxification. Alternatively, *in vitro* studies can focus on examining BPDE-DNA repair processes. This can be done by comparing nucleotide excision and base excision repair gene polymorphisms with BPDE-induced adduct formation.

Future work may also involve improving CE/LIF method sensitivity through use of a sheath flow cuvette. Also, FIA/CE/LIF analysis of standards with known adduct

concentrations will allow the determination of method sensitivity and the quantification of adducts. BPDE-DNA adduct analysis of MNCs from additional smokers will help to elucidate whether this method is suitable for biomonitoring. If the method is proven useful to detect adducts in exposed individuals, potential projects may include comparisons of MNC adduct levels to various individual cigarette smoking measures (pack years, cigarettes/day, cigarette tar content, etc), assessment of adducts in MNCs of individuals following consumption of charred meats, and/or assessment of adducts in workers exposed to diesel exhaust. Additionally, *in vivo* studies evaluating the relationship between BPDE-DNA adducts in blood and carcinogenesis may help to clarify the association between BPDE induced DNA damage and cancer.

Utilizing FIA/CE/LIF for BPDE-DNA adduct studies may help to further characterize the relationship between BaP exposure and BPDE-DNA adduct formation. This may improve our understanding of the practical application of DNA adduct data in risk assessment.