12-3-2014

Metabolic Toxicity Studies using High Throughput Bioreactor Assay and Liquid Chromatography Coupled with Mass Spectrometry (LC-MS/MS)

Dandan Li
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Dandan Li

University of Connecticut, 2014

Metabolic toxicity is one of main concerns in drug discovery and development. High efficient and low cost metabolic toxicity assessment is still a major challenge for pharmaceutical industry. This thesis focuses on the development of high throughput \emph{in vitro} metabolic toxicity assay coupling with liquid chromatography tandem mass spectrometry for metabolic adducts generation, characterization and quantitation. The key technology was based on electrostatic fabrication of polyelectrolytes and biomolecules on magnetic beads to form bioreactors in 96 well plate format and acquired metabolites induced toxicity molecular information by LC-MS experiments.

Chapter one will address the goals and significance of the metabolic toxicity assay at the early stage of drug candidate studies. It will also provide the essential background of metabolic toxicity, including metabolic enzymes function mechanism, covalent conjugates formation chemistry and consequences, and liquid chromatography mass spectrometry detection methods.

Chapter two will reveal the first discovery on metabolic genetic adducts of benzo[ghi]perylene by the cytochrome P450 enzyme/ DNA assembled biocolloid reactors generation, and followed by LC-MS/MS analysis.
Chapter three will present the high-throughput screening and quantitation assay for comparing genotoxicity profiles towards hepatic and extra-hepatic tissues, which could reveal the metabolic abilities of different organs for specific drugs. The LC-MS result is validated by comet assay.

Chapter four will describe a new approach to generate drug metabolites-protein conjugates and provide damaged site structural information by liquid chromatography mass spectrometry.
Metabolic Toxicity Studies using High Throughput Bioreactor Assay and Liquid Chromatography Coupled with Mass Spectrometry (LC-MS/MS)

Dandan Li
B.Sc. Lewis-Clark State College, 2008

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
At the University of Connecticut
2014
Approval Page

Doctor of Philosophy Dissertation

Metabolic Toxicity Studies using High Throughput Bioreactor Assay and Liquid Chromatography Coupled with Mass Spectrometry (LC-MS/MS)

Presented by

Dandan Li

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James F. Rusling

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University of Connecticut

2014
Dedicated to

My parents

Jie and Yasha
Acknowledgement

My motto is ‘Life is a journey’. Apparently achieving the Ph.D. is an important stop in this journey. Not only the research ability got enhanced, I also learn to be fearless, be strong and be humble. When I faced failure or trouble, my major advisor Prof. James Rusling always supported me and provided me the boost to proceed. He allowed me to think independently, work cooperatively and grow in my own pace. He encouraged me to attend scientific conference to present my work and communicate with other scientists, which helped me to learn things from other perspectives and enhance my communication skills. I would like to express my deepest gratitude to Dr. James Rusling for his enormous support, guidance and encouragement to let me become fearless, strong and humble in scientific research.

I am grateful to have Dr. Jing Zhao and Dr. Jie He as my thesis committee members. Dr. Jing Zhao and Dr. Jie He have been very approachable to provide suggestions for my general project. I would like to extend my gratitude to Prof. John Schenkman. He has been very helpful in giving me valuable feedbacks and suggestions throughout our collaborated projects. And Dr. You-jun Fu, thank you for all the help and guidance on LC-MS. I enjoyed the collaboration with Prof. Xiuling Lu, Dr. Choudhary Dharamainder, and Donghui Song.

I also want to thank my mentors, Dr. Linlin Zhao and Dr. Shenmin Pan for the precious mentorship and friendship. I really appreciate Dr. Greg Bishop helped me with writing and gave me suggestions whenever I asked. I am grateful to join Dr. Rusling’s group and enjoyed the cooperation and friendship with all the group members: Dhanuka, Colleen, Yun, Spundana, Karteek, Brunah, Min, Jennifer, Gayatri, Chi, Boya, Itti, Amit, Snehasis, Islam and Mohamed.

Finally, my most sincere gratitude goes to my families: my mom and dad, grandparents and my husband.
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Chapter 1

Introduction

1-1. Goals and significances

Xenobiotics are foreign chemicals towards an organism. Human pervasively access to various xenobiotics, including drugs, environmental pollutants, industrial products, food additives, etc. Some of the xenobiotics can be eliminated from body in their native form, while vast majority of them are enzymatically metabolized and biotransformed into either harmless form and excrete out through the urine, bile and feces, or reactive metabolites that could further interact with biomolecules such as DNA, protein or lipids which may induce biological toxicity. Therefore, it is very important to develop simple and comprehensive methods to study the metabolism of xenobiotics. High-throughput in vitro technologies are highly desired for comprehensive understanding the fundamental biotransformation mechanisms of xenobiotics, profiling and comparing the chemistry of metabolites adducts, predicting drug efficacy and adverse reactions, so that the potential toxicity issues can be predicted and resolved at the early stage of drug development.

After enzymatic bioactivation, xenobiotic substances normally are converted into electron rich intermediates, which could be chemically active towards nucleophilic biomolecules. When the reactive metabolites covalently bind to DNA and cause DNA damage, it’s known as
genotoxicity. If cells fail to completely repair the damaged DNA, it could lead to incorrect DNA replication, transcription and translation processes which may initiate tumor formation. DNA damage is the first stage of carcinogenesis, so it’s essential to investigate the potential genotoxicity of a drug candidate or an unknown xenobiotic substance. When the reactive metabolites conjugate with proteins, it may trigger immune system response or lose crucial functions. Therefore, reactive metabolites covalent binding towards biomolecules can potential induce various biological toxic consequences.

The goal of this thesis is to develop an *in vitro* assay for xenobiotic induced DNA and protein damage studies. The assay takes advantages of simple and clean sample preparation method using magnetic biocolloid reactor particles, and high sensitive and accurate molecule qualitative and quantitative analysis method by using liquid chromatography-tandem mass spectrometry. This assay can also enhance in high throughput format that significantly increase the screening efficiency and provide extensive information regarding to xenobiotic induced metabolic toxicity. The method may be potentially used as a robust and economic tool in drug candidates screening at the early stage of drug development.

1-2. **Xenobiotic bioactivation by Metabolic Enzymes**

The human body is involved a large amount of enzymes that can facilitate xenobiotic compounds metabolism. The basic purpose of metabolism is to increase the hydrophilicity and polarity of xenobiotic compounds that to eliminate out of the body. Metabolic enzymes can be categorized into three main groups: oxidation, reduction and conjugation enzymes. Cytochrome P450s (cyt P450s) are the major oxidation enzymes that commonly conduct monooxygenase reaction (Equation 1-1).
Besides cyt P450s, another two enzymes, Flavin-containing monooxygenase and alcohol dehydrogenase, also involve in oxidation reactions. Conjugation enzymes often catalyzed a hydrophilic moiety to the xenobiotic compound or its derivative that may help to stabilize the molecule and accelerate the excretion process. Common conjugation enzymes include glutathione S-transferases (GSTs), uridine 5’-diphospho glucuronosyltransferases (UGTs), arylamine N-acetyl transferases (NATs), sulfotransferases (SULTs) and epoxide hydrolase, etc.

1-2-1. Cytochrome P450s

Cyt P450s belong to the hemoprotein family that exhibit maximum absorption at 450 nm when it is in reduced state and inhibited by CO. They are the primary metabolic enzymes for a wide variety of xenobiotics. The nomenclature system of cyt P450 subfamilies is based on their percentage of sequence similarity. When two sequences have more than 55% overlap, they are considered in the same subfamily (e.g. P450 1A). In some subfamilies, additional number is used to denote the individual P450 isoforms for different species. For example, P450 3A4 refers to human enzymes, while rats have P450 3A2. Oxidation reaction is the predominant catalytic function for cyt P450s. They can also perform reduction, ester cleavage, ring formation and dehydration, etc.

The oxidation mechanisms of P450s can be divided into two parts: the first part is the activation of oxygen; the second part is the oxidation of the substrate. The generalized P450 catalytic cycle is showed in scheme 1-1. The first step is a substrate ‘RH’ binding to the

\[
RH + NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O + ROH
\]

Equation 1-1
ferric state iron at the distal region of the enzyme. The bound substrate induces a spin change in
the iron d5 orbital. In some cases, this binding can make reduction more thermodynamically
favorable.\textsuperscript{11,12} The second step is one electron reduction catalyzed by NADPH-P450 reductase.
The ferric state iron is reduced into ferrous state P450-substrate complex. The complex then
binds to oxygen in step three. The limiting factor in this step is the diffusion rate of oxygen in the
enzyme. The next step involves a second electron transferring to ferrous state P450 complex
which is provided by P450 NADPH reductase, ferredoxins or cytochrome b\textsubscript{5}.
\textsuperscript{13,14} The fifth step is
twice protonation to form peroxy- group and release a water molecule. The resulting electron-
deficient complex is highly reactive. It immediately abstracts a hydrogen atom, following by
structure collapsing and releasing the oxidized product ROH. There are some other possible
alternative routes for P450 catalytic cycle.\textsuperscript{15-22}

\textbf{Scheme 1-1.} Generalized P450 catalytically cycle.\textsuperscript{23}
57 human cytochrome P450 genes have been discovered. But most of them catalyze for sterols and vitamin A and D, only about a quarter of them is responsible to the majority of bioactivation of drugs and carcinogens. Among these P450s, about 90% of the catalytic activities for drug metabolism are attributed by P450 3A4, 2C9, 2C19, 2D6, 2E1 and 1A2 (Figure 1-1).

The majority of cyt P450s can be found in liver, except P450 1A1 and 1B1. However, extrahepatic tissues also consist of various P450s (Table 1-1). Individual P450s in different tissue sites can conduct various maker activities for related xenobiotic compounds. For example, the typical reaction for P450 3A4 is testosterone 6β-hydroxylation, which is a prototypic reaction. Because 6β-hydroxylation is not thermodynamically favorable, P450 3A4 has the intrinsic impact on steroid hydroxylation. Another example is benzo[a]pyrene 3-hydroxylation by P450 1A1.
P450 1A1 is an important extrahepatic P450 that is considered to be one of the major enzyme to induc

Table 1-1. *In vitro* activity of some common human cyt P450s.\(^{25}\)

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</table>

1-2-2. *Conjugation enzymes*

Major conjugation enzymes include GSTs, UGTs, NATs, SULTs and epoxide hydrolase. They normally facilitate a polar group to the substrate compound or metabolite to increase its hydrophilicity and stability that can be readily eliminated out of body.\(^{29}\) Therefore, these enzymes are active in detoxification of xenobiotics in majority cases. Inactivation exceptional cases are also found in several circumstances. For example, epoxide hydrolase plays an
significant role in converting polycyclic hydrocarbon benzo[a]pyrene into highly reactive and toxic metabolite 7,8-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydibenzo[a]pyrene (BPDE).\textsuperscript{30}

Conjugation enzymes normally require certain electrophilic adenosine containing cofactors to provide the functional groups for the conjugation reactions. As shown in figure 1-2, reduced glutathione (GSH) is the cofactor of GSTs, UDP-glucuronic acid (UDP-GlcUA) is required for UGTs, acetyl coenzyme A (CoA) is needed for NATs, and 3’- phosphoadenosine 5’-phosphosulfate (PAPS) is for SULTs.

\textbf{Figure 1-2.} Electrophilic adenosine containing cofactor compounds for conjugation enzymes. Conjugative functional groups are highlighted with colors.
GST can be found in cytosol, mitochondria and membrane-associated proteins in eicosanoid and glutathione (MAPEG). This thesis only focused on cytosolic GST due to the research interest. Human cytosolic GST is classified into 7 subfamilies including alpha, zeta, theta, mu, pi, sigma, and omega. They are expressed in a high level in liver, as well as brain, kidney and lung. The major function of glutathione S-transferase (GST) is to catalyze the conjugation of reduced glutathione (GSH) to the electrophilic center of xenobiotic substrates. GSH is a tripeptide that consists of cysteine, glutamic acid and glycine. Reduced form of GSH contains a free thiol group that acts as electron donor and form covalent conjugates. The GSH covalent conjugations dramatically increase the water solubility of the substrate compounds. Therefore, GSTs play an important role in detoxification of xenobiotics. However, GSTs may also induce high reactivity intermediates that result in toxicity. One example is the conjugation of GSH to ethylene dibromide producing a half-mustard/episulfonium ion, which is able to damage DNA and induce genotoxicity.

UGT is considered as the most important conjugation enzyme, responsible for the 90% hepatic clearance of drugs. Glucuronidation is the predominant reaction by UGT, which involves the covalent conjugation between glucuronic acid from UDP-GlcUA cofactor and a nucleophilic substrate. This reaction is based on second order nucleophilic substitution ($S_N2$) mechanism. A wide range of nucleophilic groups can be conjugated with glucuronic acid, such as carboxylic acids, aliphatic alcohols, phenols and thiols, etc. Therefore, it is not surprising that glucuronidation is served as a primary elimination pathway for various endogenous (e.g. bile acid, fatty acid) and exogenous (e.g. drugs) compounds. In some special cases, GST can also convert a drug to a toxicologically active metabolite. For example, morphine is the reactive metabolites of codeine via CYP2D6 bioactivation and exhibit analgesia effect. After
glucuronidation of morphine by UGT2B7, the product shows 100-times more potent than morphine.\textsuperscript{41}

NATs are cytosolic conjugating enzymes that can transfer an acetyl group from CoA to a receptor substrate. Human NATs are expressed into two polymorphic families, NAT 1 and NAT2, based on substrate specificities.\textsuperscript{42} NAT1 can acetylate p-aminosalicylate (p-AS) and para-aminobenzoate (pABA), while NAT2 catalyzes the inactivation of anti-tubercular agent isoniazid.\textsuperscript{42} Typical examples of conjugation reactions catalyzed by NATs include p-aminobenzoic acid acetylation, arylhydrazines of isoniazid, O-acetylation of N-hydroxy-4-aminobiphenyl and N,O acetyl transfer of N-hydroxy-2-(acetylamino) fluorene. (Figure 1-3).\textsuperscript{43} Both O-acetylation and N,O acetyl transfer reactions are associated with carcinogenic activation.

\textbf{Figure 1-3.} Typical examples of acetylation by NAT.
SULTs play an important role in xenobiotic detoxification by transferring a sulfonate moiety from PAPS to an electrophilic substrate to increase its solubility and biological stability. SULTs are also cytosolic enzymes that distributed widely in human tissues. Their target substrates normally contain hydroxyl- or amino-group. For a substrate like acetaminophen or dopamine, the sulfonation by SULTs can help them to excrete via kidney or bile. However, for xenobiotics such as N-hydroxyl arylamines or hydroxymethyl polycyclic aromatic hydrocarbons, SULTs can convert them into highly reactive electrophiles that lead to carcinogenic toxicity. The possible explanation for SULTs bioactivation activity is that sulfate group has electron-withdrawing ability to induce heterolytic cleavage, which results in the formation of a strong electrophile.

1-3. Reactive metabolites induced toxicity

After a drug or other xenobiotic entering human body, it can be processed in multiple pathways (Scheme 1-2). The drug can directly interact with a related receptor that leads to either positive effect like activating system to trigger detrimental signal, or negative effect that lose critical function. It can also undergo with metabolism pathway and be converted to its corresponding products by various metabolic enzymes. The majority of these products are water soluble and biologically stable that can be eliminated via kidney or bile, which refer to detoxification. However, some of the products are reactive electrophiles that covalently bind to a wide variety of biomolecules and potentially lead to different toxic consequences. When reactive metabolites attack DNA, the formation of DNA adducts may initiate mutation which is a potential cause of cancer. If reactive metabolites modify proteins, the damaged proteins may lose their crucial functions or activate system detrimental response which can result in apoptosis and necrosis. The conjugation of reactive metabolites and small nucleophiles (e.g. GSH) normally is a
detoxification process. Nevertheless, oxidative stress can lead to macromolecule damage with the depletion of small nucleophiles. In the following sessions, discussion will provide more insights into these metabolic toxicity effects.

![Scheme 1-2. Major biological events of drug metabolic toxicity.](image)

1-3-1. DNA Damaged by Reactive Metabolite of Xenobiotics

DNA can be damaged by either intrinsically reactive carcinogens or reactive metabolites and cause genotoxicity. To date, DNA adduct formation is considered as a common property for most carcinogens. Even though the entire process from molecular adduct initiating event to toxic outcome is complicated in biological environment, the molecular adduct is the key event to trigger the adverse outcome pathway. A large number of toxicological studies involve carcinogen-DNA adduct as measurement endpoint to establish mutagenesis metabolisms. Our discussion
in this thesis will focus on the formation of metabolites-DNA adducts, including the genotoxicity mechanisms and \textit{in vitro} covalent adduct detection techniques.

1-3-1-1. Chemistry of DNA Adduct Formation

The possible mechanisms leading to the formation of DNA adducts include seven ‘mechanistic domains’: acylation, Michael addition (MA), Schiff base formation (SB), aromatic nucleophilic substitution (SNAr), unimolecular aliphatic nucleophilic substitution (SN1), bimolecular aliphatic nucleophilic substitution (SN2), and free radical reactions (Rad).\textsuperscript{50} These mechanisms can help to assess the ability of a xenobiotic or its metabolite to act as a carcinogen, which will not be discussed in detail here.

The preferred attacking sites in deoxyribonucleosides depend on the natural of electrophiles, DNA sites reactivity and steric hindrance. The electrophiles generally indicate alkylating agents or arylaminating agents, which are generated via oxidation, reduction or conjugation reactions. The common active sites on each DNA base are highlighted in figure 1-4.\textsuperscript{47} Notably, Most of the nitrogen atoms at heterocyclic rings and oxygen atoms at exocyclic ring has relatively high reactivity. Among those sites, the nitrogen at 7 position of guanine shows the highest reactivity and is the most common attacking target for alkylating agents.\textsuperscript{47} However, the substitution for arylaminating agent is different from alkylating agent. The major conjugative site for arylaminating agent is at the C8 position of deoxyguanosine.\textsuperscript{47} The steric hindrance issue can also affect the DNA adduct formation. For example, bulky polycyclic aromatic hydrocarbon reactive metabolites, benzo[a]pyrene-7, 8-dihydrodiol 9, 10-oxide (BPDE), preferentially attack highly exposed site of deoxyguanosine at N2 position.\textsuperscript{51}
Figure 1-4. The common active sites on each DNA base with highlighted arrows.

1-3-1-2. Biological Consequence of DNA Adduct

The formation of DNA adduct can trigger specific mutations, which account for either conformational or chemical changes. Conformational interconversions attribute to the nature of the DNA adduct and the adhered bases of lesion sites. For example, the N-glycosyl bond keeps the deoxyribose sugar away from sterically bulky nucleobase as anti-conformation. However, when an aromatic amine substrate (e.g. 2-Aminofluorene (AF)) attacks C8 position of deoxyguanosine and forms a sterically hinder, it shifts this N-glycosyl bond into syn-conformation. This conformation change can cause the disruption of Watson-Crick H-bonding in duplex DNA. Meanwhile, base substitution, especially G → T mutations, may occur due to this aromatic amine DNA adduct formation. The frame shift mutation caused by AF-dG adduct is sequence-dependent and can be affected by the nearest neighbors. The conformational equilibrium of the AF-dG adduct is established among those factors. Oxidative DNA adducts, like 8-Oxo-dG and 8-Oxo-dA lesions, can interrupt base complementary interaction and conformation of single-stranded DNA. 8-Oxo-dG is preferentially paired with A and C. When pairing with A, it adopts the syn-conformation in the purine mispairs. That is account for the G→T transversion from misinsertion of dATP towards 8-Oxo-dG. On the contrary, when it pairs with C, the anti-conformation is adopted with the glycosyl bond and the resulting 8-Oxo-dG–C pair
forms Watson-Crick H-bonds.\textsuperscript{58} Polycyclic aromatic hydrocarbons (PAHs) induced DNA adducts can extensively affect the conformation of DNA helix due to their large planar fused aromatic rings. Taking benzo[a]pyrene (B[a]P) as an example, the structure-to-activity relationship of B[a]P-N2-dG adducts have been well studied.\textsuperscript{59-62} B[a]P-N2-dG adducts are found to induce mutation if without repairing.\textsuperscript{62}

However, even though N7 position of guanine is the most reactive site for electrophiles attacking, N7-guanyl adducts has small impact on biological consequences.\textsuperscript{63} Because they have relatively short half-lives and hardly trigger conformational rearrangements or bases interconversion.\textsuperscript{64} In addition, N7-guanyl adducts modified DNA can be easily recognized and repaired by endogenous repair enzymes, which will prevent further damage.\textsuperscript{65} Nevertheless, N7-guanyl adducts can still be used as a biomarker for genotoxicity studies.\textsuperscript{66}

\textbf{1-3-2. Reactive Metabolite Induced Protein Damage}

It has been almost 70 years since Elizabeth and James Miller first discovered the covalent binding between a methylaminoazobenzene carcinogen and tissue proteins.\textsuperscript{67} It remains a difficult issue for the analysis of protein covalent modification due to the low binding level and complexity of biological matrix. The relationship between covalent binding and toxic consequence is not straightforward. High level of covalent protein binding with reactive metabolites does not necessary leads to distinguishable toxicity.\textsuperscript{68} Most current \textit{in vitro} detection methods of protein adducts are costly and time-consuming, which may not fit for early toxicity assessment for drug development.\textsuperscript{69}
1-3-2-1. **Adverse Drug Reactions**

Adverse drug reactions (ADR) remain a major concern in pharmaceutical industries, around 30% of effort is contributed to ADR studies during drug development process.\textsuperscript{70} Nevertheless, ADR still a primary reason for drug withdrawing from the market. An investigation of new drugs approved on the US market between 1980 and 2009 found that 26 out of 740 new drug compounds were withdrawn due to ADR.\textsuperscript{71} ADR can be classified into five categories: ‘on-target’ toxicity, ‘off-target’ reactions, hypersensitivity and immunogenicity, idiosyncratic reactions and bioactivation induced toxicity.\textsuperscript{72} On-target toxicity normally is predictable and avoidable since it is mechanism based and dose-relative.\textsuperscript{73} Hypersensitivity and immunogenicity is associated with β-lactam antibodies which will be triggered by hypotheses ‘danger’ xenobiotics.\textsuperscript{74,75} Off-target reactions is often considered into idiosyncratic adverse reactions (IDARs) since both of them are unpredictable and host-dependent. Their mechanisms are poorly understood and difficult to control.\textsuperscript{76,77} Bioactivation of drug to reactive metabolites which covalently bind to biomolecules may subsequently trigger cell damage and induced biological toxicity. Some of bioactivation induced toxicities are considered as IADRs. This thesis will mostly focus on this aspect of ADR.
Scheme 1-3. Brief schemes of the hapten, danger and pharmacological interaction hypotheses. In the hapten hypothesis, a drug or reactive drug metabolite covalently binds to protein and form modified protein, which is taken up by antigen presenting cells (APCs) to involve in major miscompatibility complex (MHC) to helper T cell, and trigger signal 1 for immune response. In the danger hypothesis, co-stimulation of two signals is required to trigger immune response. In the pharmacological interaction hypothesis, drug directly attach to the major histocompatibility complex (MHC)-T cell receptor (TCR) complex to initial immune response.
Several mechanisms of IADRs have been proposed to explain how covalent binding of reactive metabolites triggers immune response and leads to cell damage (Scheme 1-3). First one is the hapten hypothesis. A hapten is a drug or reactive drug metabolite covalently binds to protein and result in drug-protein adduct. The immune system learns to recognize this hapten as foreign and response with immune signal. Penicillin allergies can be explain by hapten hypothesis. β-lactam ring of penicillin can actively conjugate to proteins which would lead to immune response. The second one is danger hypothesis which argues that foreign protein conjugates do not invoke immune response without an adjuvant to stimulate antigen presenting cells (APCs). This hypothesis claims that immune response is stimulated by danger signals from damaging cells. Therefore, only those drug-protein adducts that are able to trigger danger signals will cause immune response. The third one is pharmacological interaction hypothesis. In this hypothesis, the drug directly attacks to the major histocompatibility complex (MHC)-T cell receptor (TCR) complex to initial immune response without metabolism.

1-3-2-2. Liver injury (Hepatotoxicity)

Liver is the primary organ for drug metabolism. So it is not surprising that liver is also the major attack site for most reactive metabolites, which may cause hepatocytes death or trigger an immune response. Drug reactive metabolites, act as electrophiles, can covalently conjugate with protein, DNA, lipids, which will subsequently initiate oxidative stress, GSH depletion and mutation, etc. Two major types of idiosyncratic liver injury (IDILI) are hepatocellular and cholestatic. Hepatocellular liver injury is the most common IDILI to cause the death of hepatocytes. According to Hy’s law, serious IDILI of a drug is closely related to its mild IDILI, but the incident ratio between them is not clear. Hepatic damage does not necessary occur in
the centrilobular region with highest concentration of cytochrome P450s, but spread in the whole liver. Cholestatic liver injury is defined when the ratio between alanine transaminase and alkaline phosphatase is smaller than two. Cholestatic IDILI has less possibility to cause liver failure than hepatocellular IDILI, but it takes longer time to recover.

1-3-2-3. Acetaminophen (Paracetamol)

Acetaminophen (APAP) has been well-known as an analgesic and antipyretic agent for over a hundred years. But it is also responsible for 80% of drug-related liver failure cases, while about half of these cases were unintentional poisons. Hepatotoxicity study of APAP was initiated by Jollow et al. in 1973. At therapeutic does, it’s a safe drug due to the effective detoxification pathways. In cases of acute overdoes, it could lead to severe hepatic necrosis. The primary detoxification pathways of APAP metabolism are glucuronidation and sulfonation. Both of them can produce hydrophilic forms of APAP derivatives that lead to urinary excretion. Another metabolic pathway of APAP is to be oxidized into reactive intermediates, N-acetyl-p-benzoquinoneimine (NAPQI), by cytochrome P450 enzymes. NAPQI is a highly reactive two-electron electrophile, which can be efficiently detoxified by the conjugation with low-molecular-weight nucleophile reduced glutathione (GSH). Nevertheless, when GSH is depletion and detoxification capacity is saturated, accumulation of NAPQI is then free to covalently conjugate to hepatic proteins. The covalent binding of NAPQI towards cellular protein can disrupt critical enzyme activity or regulation pathways, which may further result in centrilobular hepatic necrosis.
1-4. Mass Spectrometry in Metabolite and Biomolecule Adduct Studies

There are numerous methods for xenobiotic metabolites and biomolecule damaged detection, such as isotopic labeling, immunoassay, electrochemistry assay and mass spectrometry. Mass spectrometry is a very powerful analytical technique for the characterization and quantitation of chemicals based on their ion mass-to-charge ratios. Current techniques often couple mass spectrometry with liquid chromatography (LC) or gas chromatography (GC) to increase the sensitivity and capability in complex sample analysis. GC-MS is mostly used for small volatilized compounds analysis, while LC-MS can be applied for a much wider range of chemicals. Our research is mostly employed LC-MS for analytes separation and analysis, so GCMS and other detection methods will not be included in this discussion.

1-4-1. Basics of Mass Spectrometry

The basic theory of mass spectrometry is to ionize molecules and acquire their structural information by manipulating the electric and magnetic fields. The major components for a mass spectrometer include ion source, mass analyzer, ion detector, vacuum pump and data processor (Figure 1-5).
Ionization is a crucial step in mass spectrometry analysis. Many ionization methods have been developed for different applications. In the early days, electron impact (EI) and fast atom bombardment (FAB) are two classic methods for ionization. Nowadays, with better ionization efficiency and higher sensitivity, the common techniques include electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), etc. ESI has become the most popular ionization method that is extensively used for both small chemicals and macromolecules (Figure 1-6). Generally the analytes containing volatile solvent from LC separation is injected into the inlet capillary of ESI ion source. A very high voltage is applied to induce the dispersion of the analyte solution into the spray nozzle with highly charged electrospray droplets. A nebulized gas flow can shear the analyte solution and direct the charged droplets to exit the nozzle tip. The droplets then get de-solvated by heat, nitrogen drying gas and vacuum, and become charged progeny droplets with smaller size. Finally, the electric field strength within a
droplet exceeds to a limit that allows analyte ions to eject from the droplet, and accelerate into mass analyzer by voltages. For large molecule analytes, the ions may carry multiple charges.\textsuperscript{104} MALDI is a soft ionization technique that requires a matrix to mix with the sample, then ablation and desorption is triggered by laser radiation. The analyte ions are finally formed via protonation or deprotonation. This method is especially suitable for ionizing large fragile biomolecules without damaging their structure.\textsuperscript{105}

![Figure 1-6](image)

**Figure 1-6.** A generalized schematic of ESI-ion source.

### 1-4-2. Mass analyzers

Mass analyzers manipulate ion separation according to their mass-to-charge ratio in electric and magnetic field. The dynamics of ions follow Lorentz force law and Newton's second law, which reveals that the mass-to-charge ratio of an ion is inverse proportional to its acceleration under certain electric field and vector cross product.\textsuperscript{106} There are numerous mass analyzers using either static or dynamic field of electric or magnetic power.\textsuperscript{103} The important
criterions to evaluate a mass analyzer include the mass resolving power, the mass accuracy, the mass range and the linear dynamic range.  

A quadrupole mass analyzer is most commonly used in clinical laboratories. It consists of four parallel metal rods. Oscillating electric field is created by DC voltage superimposed with radio frequency (RF) AC voltage on each diagonally placed rod pair, which allows only desired ions passing through. On the other hand, undesired ions hit the metal rod and get neutralized without reaching the detector. A quadrupole mass analyzer can act as a robust and economical mass selective filter or ion trap. When three quadrupoles consecutive set up together, they are often called as “triple-quad”. The desired ions can be selected in the first quadrupole and then fragmented by inert gas in a second RF-only quadrupole collision cell, following by resulting product ions monitoring in a third quadrupole mass analyzer. This triple quad system can provide high resolution and sensitivity capability for molecular structure and quantitative analysis.  

Time-of-Flight (TOF) mass analyzer is using an electric field to accelerate ions under the same potential, following the Lorentz force law and Newton's second law mentioned above, ions with different mass-to-charge ratios achieve different velocities. The result is measured by the time for each ion taking to reach the detector. TOF mass analyzer is often hybridized with a quadrupole to enhance the performance for wider applications.

1-4-3. Sample preparation by magnetic biocolloid reactor assay

Sample preparation is the key step in analytical methods, which is often skill-specific, labor-intense, high cost and time-consuming. One of the major aims of this thesis is developing and optimizing the magnetic biocolloid reactor assay to generate adequate and clean sample
analytes in a simple and high-throughput format. This assay is based on layer-by-layer assembly theory. Electrostatic based layer-by-layer self-assembly is a simple and universal technique to immobilize charged biomolecules onto a solid charged surface, which may increase the reaction efficiency and simplify purification process.\textsuperscript{110,111} Iron oxide based magnetic particles provide a solid support for the films even more shorten the separation and purification steps by magnetic power, comparing with non-magnetic material based particles.\textsuperscript{112} The important characteristics of electrostatic layer-by-layer fabrication are: 1) Nonlinear ‘island-like’ growth at the first couple layers that smaller amount of charged macromolecules can be absorbed;\textsuperscript{110} 2) The outer layers with linear growth provide continuous charge to help the enzyme absorption; 3) Biocolloid particles that suspended in buffer solution is able to expose effective surface area to absorb enzymes. 4) High surface concentration of biomolecules can increase the reaction efficiency. 5) Immobilization of biomolecules simplified the purification steps, avoiding some time-consuming operations like precipitation or centrifugation.

1-5. Summary

Metabolic toxicity study is an important issue for drug development and xenobiotic metabolism study. This thesis focuses on \textit{in vitro} method development and application for xenobiotic induced DNA and protein damage screening studies. Chapter two will reveal the first discovery on metabolic genetic adducts of benzo[ghi]perylene. Chapter three will present the high-throughput screening and quantitation assay for comparing genotoxicity information towards hepatic and extra-hepatic tissues. Chapter four will describe a new approach to generate drug metabolites-protein conjugates and provide damaged site structural information by the magnetic biocolloid reactors coupling with LC-MS/MS analysis.
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Chapter 2

Genotoxicity-Related Chemistry of Human Metabolites of Benzo[ghi]perylene (B[ghi]P) Investigated using DNA/Microsome Biocolloid Reactors with LC-MS/MS

2-1. ABSTRACT

There is limited and sometimes contradictory information about the genotoxicity of the polycyclic aromatic hydrocarbon benzo[ghi]perylene (B[ghi]P). Using recently developed metabolic toxicity screening biocolloid reactor-LC-MS/MS approach, featuring films of DNA and human metabolic enzymes, we demonstrated the relatively low reactivity of metabolically activated B[ghi]P toward DNA. Electro-optical toxicity screening arrays showed that B[ghi]P metabolites damage DNA at a 3-fold lower rate than benzo[a]pyrene (B[a]P), whose metabolites have a strong and well-understood propensity for DNA damage. Metabolic studies using magnetic bead biocolloid reactors coated with microsomal enzymes in 96-well plates showed that cyt P450s 1A1 and 1B1 provide high activity for B[ghi]P and B[a]P conversion. Consistent with published results, the major metabolism of B[ghi]P involved oxidations at 3,4 and 11,12 positions, leading to the formation of B[ghi]P 3,4-oxide and B[ghi]P 3,4,11,12-bisoxide. B[ghi]P 3,4-oxide was synthesized and reacted with deoxyadenosine at N6 and N7 positions and with deoxyguanosine at the N2 position. B[ghi]P 3,4-oxide is hydrolytically unstable and transforms into the 3,4-diol or converts to 3- or 4-hydroxy B[ghi]P. LC-MS/MS of reaction products from the magnetic
biocolloid reactor particles coated with DNA and human enzymes revealed for the first time that a major DNA adduct results from the reaction between B[ghi]P 3,4,11,12-bisoxide and deoxyguanosine. Results also demonstrated 5-fold lower formation rates of the major DNA adduct for B[ghi]P metabolites compared to B[a]P. Overall, results from both the electro-optical array and biocolloid reactor-LC-MS/MS consistently suggest a lower human genotoxicity profile of B[ghi]P than B[a]P.
2-2. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, and some are carcinogenic in animals and humans.\textsuperscript{1-3} PAHs are lipophilic and are normally transformed into more hydrophilic products by metabolic enzymes, such as cytochrome (cyt) P450s. Certain metabolites of PAHs (e.g., benzo[\textit{a}]pyrene 7,8-dihydrodiol 9,10-epoxide (BPDE) are highly reactive toward nucleophilic sites of biomolecules such as DNA, producing DNA adducts that may induce mutations that potentially initiate tumor formation or other diseases.\textsuperscript{4-6} Correlations of DNA adduct levels with tumor-inducing potencies exist for a number of PAHs, including benzo[\textit{a}]pyrene (B[\textit{a}]P) and dibenzo[\textit{a},\textit{l}]pyrene in rodents.\textsuperscript{7-9} These data relate to the relative toxicity of the PAH itself, and DNA adducts can serve as biomarkers for individual exposure to PAHs.\textsuperscript{10,11}

The formation of DNA-reactive PAH metabolites generally begins with oxidations catalyzed by cyt P450s.\textsuperscript{4,12,13} Oxidations can occur in the so-called K or bay regions (Scheme 2-1), producing phenols, dihydrodiols, and/or epoxides.\textsuperscript{3} Structural variations may contribute to variation in reactivity and tumorigenic potencies.\textsuperscript{14}

\textbf{Scheme 2-1.} Structures of B[\textit{a}]P (on left) and B[\textit{ghi}]P (on right) showing the Bay and K regions.
B[α]P, perhaps the most well understood PAH in terms of genotoxicity, undergoes metabolic pathways producing diol epoxide and radicals that react with DNA in vitro and in vivo\textsuperscript{15–17}. The active bay region (Scheme 2-1) diol epoxide BPDE is most significant as it forms stable DNA adducts that can lead to incorrect DNA replication and tumor initiation\textsuperscript{18,19}.

Benzo[ghi]perylene (B[ghi]P) is widely distributed in petroleum and coal tar\textsuperscript{20,21}. Its structure features two K regions and represents PAHs that lack the very reactive bay region. Formation of a vicinal diol epoxide like BPDE from B[ghi]P is not possible\textsuperscript{22}. B[ghi]P displays mutagenicity in the Ames test with exogenous activation but was negative in tumor initiating tests in mouse skin\textsuperscript{23}. Another investigation of B[ghi]P showed that active metabolites can bind to DNA in vivo and in vitro\textsuperscript{24}. Platt et al. using mouse microsomal biotransformation of B[ghi]P and 32P-postlabeling suggested that 3,4-epoxy-3,4-dihydro-B[ghi]P (B[ghi]P 3,4-oxide) might be a carcinogenic metabolite with DNA reactivity\textsuperscript{25}. Comet assays yielded DNA damage for both B[α]P and B[ghi]P when activated by light without metabolic activation\textsuperscript{26}. However, there is no conclusive evidence to classify B[ghi]P as a carcinogen in humans, nor is the chemistry of DNA damage well understood.

The goal of the present study is to establish the relative reactivity and chemistry of B[ghi]P metabolites towards DNA compared to that of B[α]P, employing in vitro metabolite toxicity screening approaches developed in our laboratory using DNA-damage as detection end points\textsuperscript{27,28}. First, an electrochemiluminescent (ECL) array utilizing human enzyme/DNA films screening metabolite reactivity with DNA was employed (Figure 2-1A)\textsuperscript{29,30}. Then, biocolloid reactor particles coated with similar enzyme/DNA films were used to produce metabolites and DNA adducts for LC-MS/MS analysis (Figure 2-1B)\textsuperscript{31,32}. Both of these approaches indicate the
formation of metabolites that can possibly react with DNA, and that may be linked to genotoxicity.\textsuperscript{27,28}

\textbf{Figure 2-1.} Methods for DNA damage detection and identification: (A) ECL screening array showing an enzyme/DNA film assembled on array spots and ECL detection utilizing a dark box equipped with a CCD camera and (B) magnetic particle biocolloid reactors and 96-well plate for high-throughput LC-MS/MS detection of DNA adducts. Incubation of assembled biocolloid reactors with damage agent and subsequent DNA hydrolysis is done in a 96-well filter plate. Solutions containing hydrolyzed products of DNA and hydrolysis enzymes are transferred into a second filter plate. A third plate is used to collect filtered samples containing the nucleoside adducts for LC-MS/MS analyses.
Both approaches use thin films of enzymes and DNA deposited on either a graphite chip for the ECL array or on 1 µm magnetic beads processed in 96-well plates for LC-MS/MS analyses. Specifically, ECL arrays feature multiple spots composed of ~40 nm thick composite films of double-stranded DNA, metabolic enzymes, and a ruthenium metallopolymer (RuPVP) that emits ECL light upon reaction with guanines in the DNA. These films are grown layer by layer via alternate electrostatic absorption.\textsuperscript{29} In the assay, parent compounds are similar enzyme/DNA films were used to produce metabolites and DNA adducts for LC-MS/MS analysis (Figure 2-1B).\textsuperscript{31,32} Both of these approaches indicate the formation of metabolites that can possibly react with DNA, and that may be linked to genotoxicity.\textsuperscript{27,28} Both approaches use thin films of enzymes and DNA deposited on either a graphite chip for the ECL array or on 1 µm magnetic beads processed in 96-well plates for LC-MS/MS analyses. Specifically, ECL arrays feature multiple spots composed of ~40 nm thick composite films of double-stranded DNA, metabolic enzymes, and a ruthenium metallopolymer (RuPVP) that emits ECL light upon reaction with guanines in the DNA. These films are grown layer by layer via alternate electrostatic absorption.\textsuperscript{29} In the assay, parent compounds are converted into metabolites that react with nucleobases in the films leading to the partial disruption of the DNA double helix. Disorder in the DNA structure from the damage reactions provides guanines that are more accessible to the RuPVP centers compared with intact DNA. Therefore, the reaction between guanine and RuPVP is accelerated, producing an enhanced ECL signal in the detection step.\textsuperscript{27–29}

The LC-MS/MS assays utilize magnetic biocolloid reactor particles coated with enzymes for metabolite profiling or with enzymes and double stranded (ds)-DNA to determine DNA adducts. These magnetic biocolloids bring the reaction components into close proximity, which greatly accelerates the production of active metabolites and DNA adducts compared with that in
conventional solution phase enzyme reactions. A magnet facilitates sample preparation in 96-well plates (Figure 2-1B). Similar to ECL arrays, active metabolites generated in enzyme reactions are initially trapped by DNA in the thin films on the magnetic beads, and DNA adducts along with intact bases are subsequently released by enzyme hydrolysis followed by LC-MS/MS analyses (Figure 2-1B). Using these approaches, we demonstrate in this article significantly lower B[ghi]P metabolite reactivity toward DNA than B[a]P metabolites and identify a new adduct of B[ghi]P from the reaction of B[ghi]P 3,4,11,12-bisoxide and deoxyguanosine.

2-3. EXPERIMENTAL SECTION

**Caution:** B[ghi]P and B[a]P are suspected human carcinogens. Procedures were done wearing gloves in a closed hood.

2-3-1. Chemicals and Materials. Human liver supersomes 1A1 (Cyt P4501A1OR) human liver supersomes 1B1 (Cyt P4501B1OR), and human liver supersomes 1A2 (Cyt P4501A2OR), all containing reductase and epoxy hydrolase, were from BD Biosciences. Carboxylated magnetic particles were from Polysciences (Warrington, PA; 1 µm diameter; particle concentration 20 mg mL⁻¹). B[a]P was from Toronto Research Chemicals. Water was purified with a Hydro Nanopure system to specific resistance >16 MΩ. B[ghi]P and all other chemicals were from Sigma-Aldrich. NADPH regenerating system: glucose 6- phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), and nicotinamide adenine dinucleotide phosphate (NADP), and other chemicals were from Sigma.
2-3-2. Films on Magnetic Particles. LbL enzyme-DNA film fabrication on 1 µm particles was similar to that reported previously.\textsuperscript{31,32,35} Briefly, polycation poly(diallyldimethylammoniumchloride) (PDDA), supersomes and salmon testes dsDNA were assembled in alternate successive steps on the negatively charged magnetic particle surface.\textsuperscript{36} Steady-state adsorption times were 20 min for PDDA and DNA solutions and 30 min for supersomes while kept on ice. After each layer of adsorption, the particles were first separated from the solution using aligned magnets, then washed, and redispersed in 10 mM, pH 7.4 Tris buffer. Final film architectures on the magnetic biocolloid reactors were PDDA/supersomes/PDDA/DNA. For the generation of PAH metabolites, final films were PDDA/supersomes.

2-3-3. Synthesis of 3,4-Epoxy-3,4-dihydro-B[ghi]P (B[ghi]P 3,4- Oxide). B[ghi]P 3,4-oxide was prepared by epoxidation of B[ghi]P with dimethyldioxirane (DMDO).\textsuperscript{37,38} To a solution of B[ghi]P (6 mg, 22 mmol) dissolved in acetone (1.5 mL) and dichloromethane (1.0 mL), DMDO (120 mmol) in 2 mL of acetone was added. After overnight stirring, the reaction mixture was dried under vacuum, and the resulting orange solid was dissolved in CDCl3 and analyzed by NMR (Figure 2-2).
Figure 2-2. 1H NMR spectrum of products of B[ghi]P DMDO reaction. Inset is the UV chromatogram corresponded to hydrolyzed product of synthesized B[ghi]P 3,4 oxide.
2-3-4. Reaction of B[ghi]P 3,4-Oxide with Nucleosides. Six milligrams of unpurified product from the aforementioned synthesis containing ∼2.4 mg B[ghi]P 3,4-oxide was dissolved in 1 mL of DMSO and 100 µL of the resulting solution was added to 1 mL of sodium phosphate buffer (pH 7.5, 10 mM) containing 1 mg of dG or dA. Reactions were stirred for 12 h at 37 °C or 70 °C, with similar product obtained, and the resultant mixture had a dark brown color. The solvent was removed by vacuum, and the residue was dissolved in 200 µL of DMSO/MeOH (1:1), filtered, and characterized by LC-MS/MS.

2-3-5. Protocols for Magnetic Biocolloid Reactors. Three 96-well plates were employed to produce either metabolites or DNA adduct samples for LC-MS/MS analysis. In general, 100 µL of biocolloid reactor particles dispersed in 10 mM phosphate buffer (pH 7.4) containing an NADPH-regenerating system was dispensed in each well in the reaction plate. Enzyme reactions were initiated in each well in the dark by adding either 1 µL of 2.5 mM B[a]P or 5 mM B[ghi]P in DMSO. The final concentration of PAHs was 25 µM for B[a]P and 50 µM for B[ghi]P. For the production of PAH metabolites, reactions were done for 10, 20, 30, and 60 min in triplicate using biocolloid reactor particles coated with PDDA/supersome films. After the reaction, 100 µL of DMSO was added to each of the reaction wells to increase the solubility of PAH metabolites in the solution phase from reactor particles. Biocolloid reactors were separated magnetically, and solutions in the reaction plate were supplemented with 1 µM internal standard 6-hydroxychrysene (final concentration) and then transferred to the second filter plate where samples were filtered. The collecting plate underneath the filter plate was used to collect the filtered sample, which was later injected to LC for analysis.
To generate PAH metabolite-DNA adducts, 1 µm magnetic particles coated with PDDA/supersomes/PDDA/DNA were used. Reactions of magnetic biocolloids and B[a]P or B[ghi]P were conducted in the reaction plate for 5, 10, 15, and 20 min in quintuplicate for each time point at 37 °C and stopped by adding 20 µL of cold acetonitrile with 2 µL of formic acid. The resulting solutions from 5 wells for the same time point were later combined. The biocolloid reactors were separated by a magnet, washed, and reconstituted in 150 µL of 10 mM Tris buffer at pH 7.4 containing 1 mM CaCl₂, 1 mM ZnCl₂, and 10 mM MgCl₂. Enzyme hydrolysis of DNA was then done in the reaction plate at 37 °C, following the previous protocol32 with slight modification. Briefly, biocolloid reactors in each well were incubated with deoxyribonuclease I (400 unit mg⁻¹ of DNA) for 3 h, followed by incubation with phosphodiesterase I (0.2 unit mg⁻¹ of DNA), phosphodiesterase II (0.1 unit mg⁻¹), and alkaline phosphatase (1.2 unit mg⁻¹ of DNA) for 12 h at 37 °C, with a plate cover. After hydrolysis, samples were spiked with 0.1 µM 7-methylguanosine as an internal standard and transferred to the filtration plate. Upon filtration, samples were collected in the collecting plates and injected into LC-MS/MS for analysis.

2-3-6. LC-MS/MS Analysis of PAH Metabolite Mixtures. Four microliters of PAH metabolites sample was injected and analyzed using a capillary Luna C18-2 column (0.5 mm × 150 mm Phenomenex) coupled with a photodiode array (PDA) detector. Separation was achieved using a gradient of ammonium acetate buffer (10 mM, pH 5.5 with 0.1% formic acid) and acetonitrile (0.1% formic acid), with acetonitrile compositions 50% for 10 min, 50%–100% for 30 min, 100% for 10 min, 100%–50% for 2 min, and 50% for 3 min at a flow rate 15 µL min⁻¹.
2-3-7. LC-MS/MS Analysis of DNA Adducts from PAH-Metabolites. A conventional LC (Waters, 2970) and a capillary LC (Waters, Capillary LC-XE) were used as previously described. A binary separation gradient composed of A, ammonium acetate buffer (10 mM, pH 5.5 with 0.1% formic acid), and B, acetonitrile (0.1% formic acid), was used. A 20 µL reaction product of B[ghi]P 3,4-oxide and a nucleosides sample were injected and analyzed using conventional LC with a Luna C18-2 column (4.6 mm × 250 mm Phenomenex) using the following gradient: 30% B for 10 min, 30%–50% B for 10 min, 50%B for 10 min, 50%–95% B for 15 min, 95% B for 10 min, 95%–30% B for 10 min, and 30% B for 5 min at a flow rate 0.8 mL min⁻¹. For metabolite-DNA adducts using magnetic biocolloid reactors, 10 µL of the adducts sample was injected and separated using capillary LC with a Luna C18-2 column (0.5 mm × 150 mm) with the following gradient: 30% B for 20 min, 30–60% B for 10 min, 60% B for 10 min, 60–100% B for 10 min, 100–30% B for 10 min, and 30% B for 10 min at a flow rate of 15 µL min⁻¹. A 4000 QTRAP (AB Sciex, Foster City, CA) mass spectrometer with Analyst 1.4 software operated in the positive ion mode was connected to the HPLC or capillary LC. Multiple reactions monitoring (MRM) and enhanced product ion (EPI) modes were done at 5000 V ion spray voltage, 60 V declustering potential, 15–35 eV collision energy (CE), and 0.15 s dwell time. From data on internal standards, a rough estimate of the detection limit is ~0.3 fmol.

2-4. RESULTS

2-4-1. Characterization of ECL Arrays and Biocolloid Reactor Particles. Amounts of biomolecules on the 1 µm magnetic particles (Table 2-1) were estimated by measuring the concentration remaining in solution after adsorption using UV absorbance and subtracting from
the initial concentration. The total amount of supersonal protein on particles was estimated using a Bradford assay. The amount of DNA was obtained based on absorbance at 260 nm.

Table 2-1. Characterization of immobilized supersomes and DNA on magnetic beads.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Metabolic enzymes (µg of protein)</th>
<th>DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDDA/1A1</td>
<td>513 ± 42</td>
<td>–</td>
</tr>
<tr>
<td>PDDA/1A2</td>
<td>463 ± 20</td>
<td>–</td>
</tr>
<tr>
<td>PDDA/1B1</td>
<td>482 ± 17</td>
<td>–</td>
</tr>
<tr>
<td>PDDA/1A1/PDDA/DNA</td>
<td>628 ± 47</td>
<td>32 ± 11</td>
</tr>
<tr>
<td>PDDA/1A2/PDDA/DNA</td>
<td>509 ± 10</td>
<td>37 ± 13</td>
</tr>
<tr>
<td>PDDA/1B1/PDDA/DNA</td>
<td>373 ± 38</td>
<td>27 ± 7</td>
</tr>
</tbody>
</table>

2-4-2. Activation of B[a]P and B[ghi]P by Human Liver Supersomes. P450s 1A1, 1A2, and 1B1 are the major isoenzymes in the oxidation of PAHs and were thus assembled in superson films on biocolloid reactor particles to facilitate metabolic conversions of B[a]P and B[ghi]P. B[a]P is oxidized by cyt P450 1A1 and 1B1 at the 7,8 and 9,10 positions, forming B[a]P 7,8-oxide (11, Scheme 2-2) and B[a]P 9,10-oxide (12). Both metabolites can be converted into BPDE (10), the ultimate carcinogen. The hydrolysis products of 11 and 12 by epoxy hydrolase are 7,8-dihydroxy-7,8-dihydro B[a]P (7,B[a]P 7, 8-diol) and 9,10-dihydroxy-9,10-dihydro B[a]P (8,B[a]P 9, 10-diol). Also, cyt P450 1A1 and 1B1 can catalyze the formation of 3-hydroxy B[a]P (9, 3-OH B[a]P). Reaction of B[a]P with the superson-biocolloids produced LC peaks with characteristic UV spectra 42 of 9, 7, or 11 and 8 or 12 (Figure 2-3). Uncertainty
Figure 2-3 Liquid chromatogram results for major B[a]P metabolites after supersomes reaction for 20 min. (A) General LC chromatographic spectrum at absorbance of 260 nm. Arrows represent peaks of 9,10-dihydroxy-9,10-dihydro B[a]P (B[a]P 9, 10 diol), 7,8-dihydroxy-7,8-dihydro B[a]P (B[a]P 7,8 diol) and 3-hydroxy B[a]P (3-OH B[a]P) with structures shown. (B) The UV spectrum of B[a]P 9, 10 diol, (C) The UV spectrum of B[a]P 7,8 diol and (D) The UV spectrums of 3-OH B[a]P.
arises due to similarities in the UV spectra of 11 and 12 and their hydrolysis products 7 and 8 that are the more likely final metabolites. They have relatively low retention times characteristic of more polar molecules, and both showed major ions of m/z 269 in positive MS mode, corresponding to molecular ions (m/z 287) losing a water molecule. Compound 9 gave m/z 269 ([M + H]+) in positive mode and m/z 267 ([M – H]−) in negative mode MS. Biocolloid reactor particles containing individual cyt P450 1A1, 1B1, or 1A2 supersomes were also used to investigate the oxidation of B[ghi]P. Using an NADPH-regenerating system, similar chromatographic profiles were observed after metabolic conversions of B[ghi]P by P450 1A1 or 1B1 (Figure 2-4). Chromatographic peaks obtained with P450 1A2 were much smaller under the same conditions. Thus, we used cyt P450 1A1 and 1B1 for subsequent investigations. Of two major metabolites detected in the LC, the UV spectrum of metabolite we denote as 14 (tR ~11 min) was in agreement with oxidations at the 3,4 and 11,12 positions of B[ghi]P, indicating the formation of B[ghi]P 3,4,11,12-bisoxide. As described above, it is uncertain whether 14 is B[ghi]P 3,4,11,12- bisoxide (13, Scheme 2-2) or the hydrolyzed product since both have the same UV spectrum.22 Compared with B[ghi]P 3,4,11,12-bisoxide, hydrolyzed product(s) are more polar with shorter retention times,22 and 14 eluted quite early (at 30% acetonitrile gradient). Therefore, it is quite possible that 14 (Figure 2-5) represents single or multiple diastereoisomers of 3,4,11,12-tetrahydroxy-3,4,11,12-tetrahydro-B[ghi]P (Scheme 2-2, 1,B[ghi]P 3,4,11,12-tetrol). The UV spectrum of the metabolite we denote as 15 (Figure 2-5, tR ~20 min) suggests 3,4 oxidation of B[ghi]P and formation of B[ghi]P 3,4-oxide.22 Because of the short retention time, we suspect this product represents diastereoisomers of 3,4-dihydroxy-3,4-dihydro-B[ghi]P (Scheme 2-2, 2,B[ghi]P 3,4- diol). MS gave m/z of 293, corresponding to molecular ions (m/z 311) losing a water molecule (Figure 2-5). Major fragmentations of 293...
were m/z 275 and 265, corresponding to the loss of a neutral water or CO from the parent. Overall, observation of metabolites 14 and 15 suggests the formation of B[ghi]P 3,4-oxide (4) and B[ghi]P 3,4,11,12-bisoxide (3). The relative formation rates of metabolites from B[a]P and B[ghi]P were characterized based on the peak area ratios relative to internal standard.

**Figure 2-4.** Peak area ratio of major PAHs metabolite (7, B[a]P 7,8 diol or 2, B[ghi]P 3,4-diol) and 1 µM 6-hydroxy chrysene after PAHs incubated with 1A1 and 1B1 assembled magnetic biocolloids at different time points ranged from 0 min to 60 mins.
Figure 2-5. Liquid chromatogram results for B[ghi]P metabolites after supersomes reaction for 20 min using absorbance at 260 nm: (A) Arrows represent peaks of compounds \(1\) and \(2\) and B[ghi]P. (B) and (C) UV spectra of compounds \(1\) and \(2\). (D) Peak area ratio of the major PAH metabolites (7, B[\(a\)]P 7,8 diol or \(2\), B[ghi]P 3,4-diol) to internal standard 6-hydroxycrysene and after PAHs incubated with 1A1 and 1B1 assembled magnetic biocolloids at different time points ranged from 0 min to 60 mins.
6-hydroxychrysene (Figure 2-4D). Faster formation of metabolites 15 and 7 was found using P450 1A1 supersomes compared to those obtained with P450 1B1 supersomes. For both isozymes, metabolite 7 of B[a]P formed at a faster rate than metabolite 15 of B[ghi]P, i.e., 0.0037 µM·min⁻¹ for 7 and 0.0060 µM·min⁻¹ for 15 under P450 1A1 catalysis; and 0.0008 µM·min⁻¹ for 7 and 0.0024 µM·min⁻¹ for 15 under P450 1B1 catalysis. These results also suggest that the reactions proceed for up to 60 min and do not provide any indication of enzyme inhibition.

2-4-3. Nucleoside Adducts of B[ghi]P 3,4-Oxide. The detection of 15 as a metabolite of B[ghi]P and the observation of reactive metabolite-derived DNA in ECL results (vide supra) as well as previous results with mouse microsomes25 suggested the formation of 4, B[ghi]P 3,4-oxide. To help elucidate possible DNA adduct formation by 4, we synthesized it, obtaining a final product mixture of 40% B[ghi]P 3,4-oxide and 60% unreacted B[ghi]P as demonstrated by NMR (Figure 2-2). B[ghi]P 3,4-oxide was then reacted with dA or dG for 12 h to obtain the nucleoside adducts. A surrogate scan using a total ion chromatogram was used to survey nucleoside adducts formed. An enhanced product ion (EPI) scan provided collision-induced dissociation (CID) spectra of selected ions and was used for structural elucidation. Multiple reactions monitoring (MRM) revealed peaks representing mass transitions from precursor to product ion pairs. Total ion chromatograms (TIC) were obtained of all eluents containing molecular ions of m/z 544, 428, and 293 for the reaction of B[ghi]P 3,4-oxide with dA (Figure 2-6A), and of m/z 560 and 293 for the reaction of B[ghi]P 3,4-oxide with dG (Figure 2-6B). CID spectra of eluents at 33, 48, and 49 min (Figure 2-7B to D) presented major product ions, i.e., 275 and 265, of m/z 293. Therefore, these eluents most likely are transformation products of
metabolite 2 derived from the hydrolysis of 4. Previous work on benzo[e]pyrene 3,4-oxide revealed transformation into phenols 3-hydroxy and 4-hydroxy benzo[e]pyrene.\textsuperscript{45,46} By analogy, we suspect that the two eluents with longer retention times (tR) were most likely the B[ghi]P monohydroxy phenols, 5 and 6 (m/z 293) (Scheme 2-2). Eluent with tR $\sim$33 min is either 4 or hydrolyzed product 2. This observation suggests the formation of 5 and 6 from the nonenzymatic hydrolysis of B[ghi]P 3,4-oxide. All these eluents have major mass transitions of m/z 293$\rightarrow$275 and extracted SRM chromatograms of m/z 293$\rightarrow$275 (Figure 7A). Since m/z 293 ions were from either B[ghi]P 3,4-oxide or its derivatives, molecular ions of m/z 544, 428 (Figure 2-4A), and 560 (Figure 2-6B) were possible adducts produced by the reaction of B[ghi]P 3,4-oxide with dA or dG. The CID spectrum in Figure 2-4G shows product ions of m/z 544 (dA adducts), including m/z 428, 410, 293, and 136 (fragmentation pattern illustrated in Figure 2-4H). The product ions of m/z 560 (dG adducts) were m/z 444, 427, 393, and 293, as shown in Figure 2-4J. Both CID spectra show major product ions resulting from the parent ions losing 116, such as 428 from parent 544 and 444 from parent 560. A neutral loss of 116 is the fingerprint fragmentation of stable DNA adducts at low collision energy (CE).\textsuperscript{31,32} In addition, the MW of ions m/z 544 ([M + H]$^+$) matches the sum up of B[ghi]P 3,4-oxide (m/z 292) and dA (m/z = 251). Similarly, the MW of ions m/z 560 ([M + H]$^+$) matches the sum of B[ghi]P 3,4-oxide (m/z 292) and dG (m/z = 267). Therefore, it is highly likely that the m/z 544 or 560 ions are exocyclic DNA adducts derived from B[ghi]P 3,4-oxide attacking the N6 position of dA (Figure 2-4H, 18) or N2 position of dG (Figure 2-6K, 19). These positions are usually prone to reaction with bulky
Figure 2-6. LC-MS/MS analysis of reactions of synthesized \( \text{B[ghi]} \)P 3,4-oxide with dA and dG. (A) TIC containing ions of \( m/z \) 544, 428 and 293 for reaction between \( \text{B[ghi]} \)P 3,4-oxide and dA. (B) TIC containing ions of \( m/z \) 460, 444 and 293 for the reaction between \( \text{B[ghi]} \)P 3,4-oxide and dG. (C) Representative MRM chromatogram with mass transition \( m/z \) 428→293. (D) Product ion spectrum of \( m/z \) 428 at CE 25 eV. (E) Possible structures of \( \text{B[ghi]} \)P 3,4-oxide dA adducts \( m/z \) 428 where reactions happen at N\(^3\) of dA (11) and N\(^7\) or dA (12). (F) Representative MRM chromatogram with mass transition \( m/z \) 544→428. (G) Product ion spectrum of \( m/z \) 544 at CE 15 eV. (H) Possible structure of \( \text{B[ghi]} \)P 3,4-oxide dA adduct \( m/z \) 544 where reaction happens at N\(^6\) of dA (13). (I) Representative MRM chromatogram with mass transition \( m/z \) 560→444. (J) Product ion spectrum of \( m/z \) 560 at CE 15 eV. (K) Possible structures of \( \text{B[ghi]} \)P 3,4-oxide dG adduct \( m/z \) 560 where reaction happens at N\(^2\) of dG (14).
PAH metabolites.\textsuperscript{3,6} This assumption was strengthened by the common product ions m/z 293, which corresponds to 3,4-dihydro B[ghi]P 3-ol, derived from loss of either dA or dG from their parent ions. (Figure 2-6H and K). Considering products of m/z 544 ions, m/z 410 represents neutral water loss from m/z 428, and m/z 136 corresponds to an adenine. The product ion m/z 393, from m/z 560, was not a feature ion, and the structure was not identified. The fingerprint MRM spectra for these adducts were m/z 544$\rightarrow$428 for dA and m/z 560$\rightarrow$444 for dG (Figure 2-6F,I).

In addition to peaks for exocyclic dA and dG adducts of 4, two minor peaks m/z 428 with tR $\sim$ 20 min (black arrows, Figure 2-6A) were also observed. Intensities are much lower than m/z 544, indicating relatively low abundance. Fragmentation of m/z 428 yielded major ions of m/z 293 and m/z 136, and minor ions of m/z 410 and m/z 275 (Figure 2-6D; fragmentation is illustrated in Figure 2-6E). These ions match the m/z of moieties, 3,4-dihydro B[ghi]P 3-ol (m/z 293), adenine (m/z 136), B[ghi]P (m/z 275), and the water loss of parent 428 (m/z 410). Therefore, we concluded that the m/z 428 belongs to depurinated labile adducts of dA47 (Figure 2-6E, 16 and 17) that are likely formed at N3 and/or N7 positions of dA. No m/z 444 was detected correlating to the labile N7-dG adducts. The major transition for depurinated adducts was m/z 428$\rightarrow$293 (Figure 2-6C). The structures of compounds 16, 17, 18, and 19 (Figure 2-6) are shown for nucleoside alkylating the 4-position of B[ghi]P 3,4-oxide (diastereoisomers not shown). Corresponding isomers with nucleosides alkylating the 3-position (structures not shown) may cause the split peaks in the chromatograms. Major product ions and mass transitions are summarized in Table 2-2 and were used to search for B[ghi]P- derived DNA adducts in subsequent reactions with DNA–enzyme biocolloid particles as described in the following section.
Figure 2-7. LC-MS/MS analysis of B[ghi]P 3,4-oxide after reaction with dG. (A) Representative MRM chromatogram with mass transition \( m/z \) 293\( \rightarrow \)275 (B), (C) and (D) Product ion spectrum of \( m/z \) 293 at CE 35 eV for elution 2, 5 and 6.

Table 2-2. Mass spectrometry methods can used for detection of B[ghi]P 3,4-oxide nucleosides adducts.

<table>
<thead>
<tr>
<th>Targets</th>
<th>MRM</th>
<th>MS Methods (ESI⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dA Adducts</td>
<td>544( \rightarrow )428, 428( \rightarrow )293</td>
<td>EPI 544, 428</td>
</tr>
<tr>
<td>dG Adducts</td>
<td>560( \rightarrow )444</td>
<td>Major Daughter Ions 428, 293, 275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPI 560, 444</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Major Daughter Ions 444, 293</td>
</tr>
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</table>
2-4-4. Detection of DNA Adducts of PAH Metabolites. As described above, cyt P450s 1A1 and 1B1 generate larger quantities of DNA-reactive B[a]P and B[ghi]P metabolites than cyt P450 1A2. Therefore, supersomes 1A1 and 1B1 were used in calf thymus DNA/enzyme biocolloid reactions to generate DNA adducts. B[a]P and B[ghi]P were reacted separately with magnetic PDDA/supersomes/PDDA/DNA biocolloids using NADPH regeneration in 96-well reaction/ filtration plates, with enzyme hydrolysis to prepare the DNA for LC-MS/MS analysis. The active diol epoxide metabolite of B[a]P, BPDE, can react with both dA and dG in DNA forming stable adducts BPDE-dA and BPDE-dG.6 In ESI+ mode, molecular ions of BPDE-dA and BPDE-dG are m/z 554 and 570, structures 20 and 21 (Figure 2-8). Fragmentation of these adducts yielded a high intensity of product ions m/z 257 other than deglycosylated ions (m/z 438 or m/z 454). The m/z 257 ions, corresponding to 7,9-dihydro-8H cyclopenta[2,1-b]pyren-8-one, derive from ions of m/z 285 that result from sequential loss of the nucleosides and two water molecules. Therefore, the dominant mass transitions m/z 554→257 and m/z 570→257 were used to monitor the formation of BPDE-dA and BPDE-dG. EPI modes of product ions m/z 554 and m/z 570 were also employed to verify adduct structures. Figure 2-8A–D confirms two major DNA adducts with mass transitions of m/z 554→257 and m/z 570→257 observed when DNA/enzyme biocolloids reacted with B[a]P using supersomes 1A1 and NADPH. Similar data using supersomes 1B1 are shown in Supporting Information, Figure 2-9. The product profiles of molecular ions m/z 554 and m/z 570 in
Figure 2-8. LC-MS/MS analysis of reactions of magnetic biocolloid reactors with B[α]P and B[ghi]P using 1A1 supersomes. (A and C) Representative SRM chromatogram with mass transitions m/z 554→257 and m/z 570→257 indicating the formation of BPDE-dA and BPDE-dG adducts after 20 min of reaction followed by enzyme hydrolysis using supersomes 1A1. (B and D) Product ion spectrum of m/z 554 and m/z 560 at CE 30 eV. (E) Representative SRM chromatogram with mass transition m/z 593→311 indicating the formation of the B[ghi]P derived dG adduct, possibly formed from B[ghi]P 3,4,11,12-bisoxide after 20 min of reaction followed by enzyme hydrolysis using supersomes 1A1. (F) Product ion spectrum of m/z 593 at CE 30 eV. (G and H) Blue curves reflect the peak area ratios to the internal standard versus time for the sum of both BPDE-dA and BPDE-dG formation (enzymes are as indicated), and red curves represent the ratio for B[ghi]P 3,4,11,12-bisoxides-dG formation. Possible structures of BPDE-dA (20), BPDE-dG (21), and B[ghi]P 3,4,11,12-bisoxides-dG (22) are also shown with red arrows indicating the major cleavage sites.
Figure 2-9. (A) Formation of highly reactive BPDE carbenium ions which is followed by SN1 reaction mechanism to form DNA adducts. (B) Formation of B[ghi]P 3,4-oxide which is followed by SN2 reaction mechanisms to form DNA adducts. Nu stands for nucleophiles such as DNA bases.
Figure 2-8B, D demonstrate that the ions originated from a reaction of BPDE and nucleosides since both deglycosylated products, m/z 438 and m/z 454, were observed along with adenine and guanine residues (m/z 136 and m/z 152). The same mass transition was not observed when biocolloid reactors were incubated with B[a]P alone, indicating that bioactivation was necessary for the formation of BPDE nucleoside adducts. The blue curves in Figure 8G and H represent the total BPDE-DNA adduct peak area of m/z 554→257 and m/z 570→257 mass transition relative to that of the internal standard (7-methylguanosine, m/z transition 298→166). B[ghi]P 3,4,11,12-bisoxides-dG formation is also expressed as a ratio to the internal standard. For a 20 min reaction of B[ghi]P with biocolloid enzyme/ DNA reactors, no peaks were found to match the mass transitions listed in Table 2-2, suggesting little formation of DNA adducts of B[ghi]P 3,4-oxide. According to previous work, B[ghi]P 3,4,11,12-bioxides can hydrolyze to form compound 3 (diastereoisomers not shown). Experiments were done to investigate possible B[ghi]P 3,4,11,12-bisoxide DNA adducts, including (1) monitoring the signature neutral loss 116 of the sugar if exocyclic adducts are formed, and (2) scanning precursor ion (PIS) containing a fragment of m/z 293, 275 (the feature B[ghi]P and B[ghi]P 3(or 4)-ol ions). Neutral loss produced no results, but PIS showed that m/z 593 ions produced m/z 293 and 275 fragments. Molecular ion m/z 593 correlates to the conjugated product of compound 3 (MW 326) with dG (MW 267), presumably derived from B[ghi]P 3,4,11,12-bisoxide (22, Figure 2-8), although stereoisomers are possible. The CID spectrum of m/z 593 (Figure 2-8F) showed major fragments contained B[ghi]P 3,4-diol ([M + H]+ = 311) and B[ghi]P 3,4-oxide ([M + H]+ = 293), indicating that m/z 593 is a derivative of the reaction product of B[ghi]P metabolite(s) with dG. The major SRM transitions 593→311 was used for the following relative quantitation.
Relative formation rates of DNA adducts derived from B[a]P (570→257 and 554→257) and B[ghi]P (593→311) were estimated using SRM peak area ratios vs the internal standard, 7-methylguanosine (298→166) (Figure 2-8G,H). Red curves showed increases in the relative amount of B[ghi]P derived dG adduct with reaction time. Compared with BPDE-DNA adducts (Figure 2-8G–H, blue curves), B[ghi]P metabolism produced only about 20% of the BPDE-DNA adducts in 20-min enzyme reactions for 1A1 and 1B1 supersomes. This observation is consistent with ECL array results, suggesting that DNA-reactive B[ghi]P metabolites are produced at lower levels than those of B[a]P.

2-5. DISCUSSION

The results described above demonstrate that ECL genotoxicity arrays with follow up biocolloid reactor metabolite-DNA adduct analysis by LC-MS/MS are a powerful combination to help elucidate complex genotoxicity-related chemical pathways. High throughput features facilitate comprehensive investigations of metabolite reactivity with DNA. Toxicity profiles of the PAHs were initially provided by ECL arrays, then LC-MS/MS analysis of products from enzyme- and DNA/enzyme- biocolloid reactor beads provided metabolite profiling and structures and formation rates of important metabolites and DNA adducts. The results also revealed for the first time that a major human DNA adduct may result from a reaction between B[ghi]P 3,4,11,12-bisoxide and deoxyguanosine. ECL array results suggested a 3.5-fold faster metabolic bioactivation of B[a]P toward DNA damage than B[ghi]P using human cyt P450 1A1, 1B1, and 1A2 (Figure 2-4). The bioreactor- LC-MS/MS approach validated more reactive B[a]P metabolites than B[ghi]P metabolites, and relative formation of major DNA adducts of B[a]P metabolite BPDE was ~5 times faster than that of the DNA adducts of B[ghi]P metabolites. The
biotransformation of B[a]P involves three important metabolites (Scheme 2-2, blue brackets) B[a]P radical, BPDE, and B[a]P 7,8 quinone, which all react with DNA.\textsuperscript{15,16,44,49} B[ghi]P is presumably oxidized mainly at 3,4 or 11,12 positions to form K region epoxides and the active metabolites B[ghi]P 3,4-oxide (4) and B[ghi]P 3,4,11,12-bisoxide (13) that may react with DNA.\textsuperscript{22,25} Metabolites detected using enzyme biocolloid reactors and LC-MS/MS are consistent with K region epoxidation of B[ghi]P. Compounds 1 and 2, the hydrolyzed products of 3 and 4, were the two major products found when B[ghi]P reacted with cyt P450 1A1 and 1B1 (Figure 2-5). This is consistent with previous results using induced mouse liver microsomes.\textsuperscript{22} In addition to K-region epoxidized metabolites, Platt et al. also observed phenol and quinone metabolites.\textsuperscript{22} The difference from our results is most likely related to species differences since human supersomes were used in our study, and induced rat liver microsomes were used in Platt’s work. We found that human cyt P450s 1A1 and 1B1 are mainly responsible for K-region epoxidation and catalyzed faster metabolism than cyt P450 1A2, similar to B[a]P.\textsuperscript{34} However, other enzymes that metabolize B[ghi]P cannot be ruled out.

Using 32P-labeling and chromatography, Platt’s group revealed several DNA adducts including a major adduct generated from the reaction of B[ghi]P 3,4-oxide and DNA when B[ghi]P was activated by induced rat liver microsomes.\textsuperscript{25} In our work, synthetic B[ghi]P 3,4-oxide was used to confirm the generation of dG and dA adducts by LC-MS/MS (Figure 2-6), which were consistent with Platt’s observations. Our structural information from LC-MS/MS (Figure 2-6) indicated that major stable B[ghi]P 3,4-oxide DNA adducts are most likely to form at positions N2 of dG or N6 of dA. Each individual peak in the spectra may possibly represent the stereoisomers of the corresponded adduct as they would possess the same m/z and fragmentation pattern. Possible structures of these adducts were presented.
In the ECL arrays, BPDE, the active metabolite of B[a]P (Figure 2-3) reacts with dA and dG in the DNA/microsome films to induce a significant ECL increase. Formation of the corresponding DNA adducts was confirmed by LC/MS-MS (Figure 2-8A–D). In general, increases in ECL intensities correlate well with increasing amounts of BPDE dA and dG adducts observed by LC-MS/MS (Figures 2-6B and 2-6G, H). Although B[ghi]P 3,4-oxide was able to attack DNA form stable dA and dG adducts (Figure 2-6C–J), the formation of those DNA adducts from B[ghi]P 3,4-oxide generated by human cyt P450 supersomes was not found in LC-MS/MS experiments. However, adducts derived from the reaction between B[ghi]P derived bisoxides, possibly B[ghi]P-3,4,11,12-bisoxide, and dG were detected (Figure 2-8E–F). The observed B[ghi]P-3,4,11,12-bisoxide-dG adduct is likely to be the stable adduct according to its m/z. However, the LC-MS/MS method utilizing magnetic bioreactors may not detect depurinated, unstable PAH-DNA adducts as these unstable adducts can detach from the DNA on the bioreactors and be lost during washing. Compared with the total dA and dG adducts formed from BPDE, B[ghi]P-3,4,11,12-bisoxide resulted in a lower rate of DNA adduct formation (Figure 2-8G,H), indicating relatively less reactivity or smaller formation rate of B[ghi]P-3,4,11,12-bisoxide. This result is consistent with in vivo experiments in which B[ghi]P was not found to initiate tumors in mouse models.23

Undetectable B[ghi]P 3,4-oxide adducts imply very low quantities of such adducts formed in the human enzyme/DNA bioreactor system. Reasons for this may include (a) the hydrophobicity and structurally hindered nature of B[ghi]P 3,4- oxide, (b) the lack of a route featuring carbenium ions (Supporting Information, Figure 2-9) that can form with BPDE, whereas B[ghi]P can only undergo epoxidation at the 3,4 position,50 and (c) rapid hydrolysis of B[ghi]P 3,4-oxide to 2 (Scheme 2-2) and conversion to phenols 5 and 6 (Figure 2-9) in
competing reactions to adduct formation. In summary, ECL genotoxicity arrays and human enzyme/ DNA bioreactor LC-MS/MS studies were used to rapidly elucidate differences in genotoxic chemistry featuring metabolite-induced DNA damage. Our findings confirm a considerably lower genotoxic profile of B[ghi]P than B[a]P and identified a new DNA adduct of human B[ghi]P metabolism. Clearly, improvements in LC-MS/MS sensitivity could provide the detection of lower abundance metabolites and nucleoside adducts are currently being pursued in our laboratory.
2-7. REFERENCES


(22) [http://monographs.iarc.fr/ENG/Classification/index.php](http://monographs.iarc.fr/ENG/Classification/index.php)


Chapter 3

Organ-Specific Metabolic Toxicity Chemistry from Electro-Optical Enzyme/DNA Arrays and LC-MS/MS

3-1. ABSTRACT

Human toxic responses are very often related to metabolism. Liver metabolism is traditionally studied, but other organs also convert chemicals and drugs to reactive metabolites leading to toxicity. When DNA damage is found, the effects are termed *genotoxic*. Here we describe a comprehensive approach to evaluate chemical genotoxicity pathways from metabolites formed in-situ by a broad spectrum of liver, lung, kidney and intestinal enzymes. DNA damage rates are measured with a microfluidic array featuring a 64 nanowell chip to facilitate fabrication of films of DNA, electrochemiluminescent (ECL) detection polymer [Ru(bpy)$_2$(PVP)$_{10}$]$^{2+}$ {($PVP = poly(4$-$vinylpyridine)$)} and metabolic enzymes. First, multiple enzyme reactions are run on test compounds, then ECL light related to the resulting DNA damage is measured. A companion method facilitates reaction of target compounds with DNA/enzyme-coated magnetic beads in 96 well plates, after which DNA is hydrolyzed and nucleobase-metabolite adducts are detected by LC-MS/MS. The same organ enzymes are used as in the arrays. Outcomes revealed nucleobase adducts from DNA damage, enzymes responsible for reactive metabolites (e.g. cyt P450s), influence of bioconjugation, relative dynamics of enzymes suites from different organs, and pathways of possible genotoxic chemistry. Correlations between DNA damage rates from the
cell-free array and organ-specific cell-based DNA damage were found. Results illustrate the power of the combined DNA/enzyme microarray/LC-MS/MS approach to efficiently explore a broad spectrum of organ-specific metabolic genotoxic pathways for drugs and environmental chemicals.
3-2. INTRODUCTION

Toxicity assessment is a major problem in drug and environmental chemical development. This has been well documented in the drug industry where poor preclinical and clinical safety assessment correlations\textsuperscript{1,2,3,4} can be due to \textit{in vitro} models that do not broadly mimic human metabolism, distribution and toxicity.\textsuperscript{5} Currently, \textasciitilde{}1/3 of drug candidates fail due to unpredicted toxicity that is not revealed until clinical testing, after the candidate has been sent forward on the basis of \textit{in vitro} and animal test results.\textsuperscript{3,6} Toxicity bioassays or animal tests are important components of human toxicity assessment, but rarely address specific chemical pathways of toxicity. Thus, there is a critical need for bioanalytical platforms to establish the chemistry of metabolic toxicity pathways to augment traditional bioassays.

Metabolites are more often involved in toxicity-related chemical reactions than the parent compounds,\textsuperscript{7,8} and most toxicity assays include a metabolic component. While standard \textit{in vitro} bioassays historically rely on liver metabolism, extra-hepatic tissues can also metabolize xenobiotics to reactive metabolites that react with biomolecules and lead to toxic responses.\textsuperscript{9} Recent research efforts have been directed towards tissue-based organ toxicity assessment. Using tissue slices from human organs, a 2002 report found that liver, lung, intestine and kidney can all contribute to the overall capacity of xenobiotic metabolism.\textsuperscript{10} Tissue systems have drawbacks including metabolic inconsistencies, deterioration, and specialized operator skill requirements. Nevertheless, promising high-throughput commercial bioassays for safety assessment are emerging.\textsuperscript{11-13} A metabolizing enzyme toxicology assay chip (MetaChip) integrating drug metabolic toxicity and high-throughput cell-based screening was developed for anticancer chemotherapeutics.\textsuperscript{14} The integrated Discrete Multiple Organ Co-culture (IdMOC\textsuperscript{®}) array uses co-cultured cells from different organs as physically separated entities interconnected by an
overlying culture medium. Microfluidic “organ-on-a-chip” devices are being developed for high-throughput screening of drug toxicity. Despite significant progress of these tissue-based in vitro tools, variable metabolic activity of cell lines, limited lifespan and low levels of metabolic enzymes need to be addressed. In addition, most of these systems rely on measuring external metabolic biomarkers such as glucose, folate, vitamin B₁₂ and lactate, and specific pathways of toxic reactions are difficult to address.

The label genotoxic denotes compounds or their metabolites that induce genetic damage. Tests for genotoxicity involve in vitro and in vivo measurement of DNA nucleobase adducts formed by reaction with metabolites, and these adducts are effective biomarkers for pollutant exposure. We recently developed a fluidic 64-microwell chip for electrochemiluminescent (ECL) detection of DNA-damage. The chip features 20-50 nm thick films of DNA, metabolic enzymes and ECL generating metallopolymers Ru(bpy)₂(PVP)₁₀²⁺ ([PVP = poly(4-vinylpyridine)]) residing in printed nanowells on a pyrolytic graphite substrate housed in a fluidic chamber. In the first step of the assay, test compound solution is pumped over the nanowells to generate reactive metabolites, causing reactions with DNA in the films. Metabolite-nucleobase adduct formation disrupts the DNA double helix, making guanine bases more accessible to oxidation by catalytic Ru³⁺PVP sites in the measurement step. This results in larger ECL signals for damaged DNA than for intact DNA. Guanines on the DNA act as co-reactants in the ECL process when Ru²⁺PVP is oxidized to Ru³⁺PVP. A complex sequence of redox reactions provides electronically excited Ru²⁺PVP* that decays to ground state by emitting visible light. This ECL light is detected in the measurement step by a CCD camera. In general, rates of ECL signals that increase with enzyme reaction time correlate well with formation rates
of individual nucleobase adducts measured by LC-MS, and with toxicity bioassays and rodent genotoxicity metrics.$^{4,22,25}$

We also developed a high throughput LC-MS/MS companion method to determine molecular structures and formation rates of individual metabolite-nucleobase adducts.$^{26}$ The approach involves magnetic biocolloid reactor beads coated with enzyme/DNA films analogous to those in the ECL array to generate reactive metabolites and DNA damage. Reactions are run in a 96-well filter plate, followed by hydrolyzing the damaged DNA, filtering, and determining damaged nucleobase products by LC-MS/MS.

In this paper, we describe the first high-throughput ECL array and LC-MS/MS platforms designed to assess organ-specific genotoxicity chemistry pathways (Scheme 3-1). Specifically, ECL arrays and magnetic beads for LC-MS/MS were equipped with representative suites of metabolic enzymes from liver, lung, intestine and kidney to simultaneously elucidate detailed organ-specific metabolic DNA damage chemistry. Metabolic enzyme sources include organ-specific microsomes (insoluble tissue fractions), cytosols (soluble tissue fractions) and supersomes, which are recombinant enzyme manifolds of single cytochrome P450 (cyt P450) enzymes.$^{8,27,28}$ In this 2-tier analytical strategy, the ECL array first establishes relative DNA damage rates and distinguishes which metabolic enzymes from which human organs are mainly responsible for metabolism and DNA damage of a given compound. Guided by this information, individual metabolite-nucleobase structures and formation rates are then determined by LC-MS/MS from the biocolloid reactor bead studies to establish molecular pathways for DNA damage. This approach provides results to develop a detailed, comprehensive picture of genotoxicity chemistry linked to individual human organs. With these methods, known genotoxic agents 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK), 2-
acetylaminofluorene (2-AAF), and styrene revealed organ-based differences in rates of DNA damage, major metabolite-nucleobase adducts and their formation dynamics were identified, and good correlations between cell-free DNA damage in these studies with cell-based DNA damage Comet assays were found.

**Scheme 3-1:** Devices used for high-throughput analysis of organ-specific DNA damage, (a) reactive metabolite-nucleobase adduct quantitation by biocolloid reactors in 96-well plate and LC-MS/MS; (b) ECL microwell chip for DNA damage detection; (c) schematic representation of reactive metabolite-DNA adduct generation; (d) ECL fluidic chip consists of a flow cell, pyrolytic graphite chip on which 64 analytical spots containing DNA, metabolic enzymes and light emitting polymer (Ru^{II}PVP) have been fabricated.
3-3. EXPERIMENTAL

3-3-1. Chemicals and materials. Styrene (M\textsubscript{W}= 104.15), 2-Acetylaminofluorene (2-AAF, M\textsubscript{W}= 223.27), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, M\textsubscript{W}= 207.23), poly(diallyldimethylammonium chloride) (PDDA, average M\textsubscript{W}= 100,000-200,000), poly(sodium 4-styrenesulfonate) (PSS, average M\textsubscript{W}= 70000), calf thymus DNA (Type I) and all other chemicals were from Sigma. Pooled male human liver microsomes (Liver, 20 mg mL\textsuperscript{-1} in 250 mM sucrose) contained (a) 20 mg mL\textsuperscript{-1} total protein content, (b) total cyt P450 content of 340 pmol mg\textsuperscript{-1} of protein using the method of Omura and Sato\textsuperscript{29}; baculovirus-insect cell expressed cyt P450 1B1 supersomes (cyt P450 1B1), 4.5 mg/ml in 100mM potassium phosphate buffer of pH 7.4 with representative total cyt P450 content of 220 pmol mg\textsuperscript{-1} of protein; baculovirus-insect cell expressed cyt P450 1A1 supersomes (cyt P450 1A1), 5.0 mg/ml in 100mM potassium phosphate buffer of pH 7.4 with representative total cyt P450 content of 120 pmol mg\textsuperscript{-1} of protein; baculovirus-insect cell expressed cyt P450 3A4 supersomes (cyt P450 3A4), 5.0 mg/ml in 100mM potassium phosphate buffer of pH 7.4 with representative total cyt P450 content of 200 pmol mg\textsuperscript{-1} of protein; and baculovirus-insect cell expressed cyt P450 3A5 supersomes (cyt P450 3A5), 14 mg/ml in 100mM potassium phosphate buffer of pH 7.4 with representative total cyt P450 content of 1000 pmol mg\textsuperscript{-1} of protein; were from BD Gentest (Woburn, MA). Human lung microsomes (Lung), 10 mg mL\textsuperscript{-1} in 250 mM sucrose; Human intestinal microsomes (Intestine), 20 mg mL\textsuperscript{-1} in 250 mM sucrose; Human kidney microsomes (Kidney), 10 mg mL\textsuperscript{-1} in 250 mM sucrose; Human liver cytosol (HLC), 20 mg mL\textsuperscript{-1} in 50 mM Tris 150 mM KCl, 2 mM EDTA of pH 7.5; Human lung cytosol (HLuC), 12.1 mg mL\textsuperscript{-1} in 250 mM sucrose; Human intestinal cytosol (HIC), 11.7 mg mL\textsuperscript{-1} in 250 mM sucrose; Human kidney cytosol (HKC), 10.6 mg mL\textsuperscript{-1} in 250 mM sucrose were purchased from Celsis (Chicago, IL).
3-3-2. DNA-metabolite adduct quantitation by LC-MS/MS. LbL films of enzymes, DNA, PDDA with film architecture of PDDA/Enzyme/PDDA/DNA were grown on 1 mm carboxylate-functionalized magnetic beads (0.4 mg) in 10 mM Tris buffer (200 mL, pH 7.0) to make the biocolloid reactors. Beads were then incubated from 1 hr (styrene) to 18 hr (NNK) with an NADPH regeneration system at 37 °C to generate metabolites. Reaction times were chosen empirically to achieve sufficient amounts of nucleobase adducts for analysis. DNA adducts formed on beads were hydrolyzed enzymatically and/or thermally. A Waters Capillary LC-XE with trap column interfaced with a 4000 QTRAP (AB Sciex) mass spectrometer was operated in positive ion mode. Estimated detection limit for nucleobase adducts is ~0.3 fmol.

3-3-3. Comet assays. A549, Caco-2, HEK 293 and Hep G2 cells (5x10^4 cells/well) were seeded on 12-well plates and cultured for 24 h at 37°C and 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM, 1 mL/well) containing 10% (v/v) fetal bovine serum, 2mM L-glutamine, 100 U/mL of penicillin and 0.1 mg/mL streptomycin. A monolayer of cells was then treated with 150 µM of the test compound 37°C for 24, 36, 48 and 60 hours. The cells were harvested and resuspended in Ca- and Mg-free PBS for the Comet Assay.

Comet assay was run by using OxiSelect™ Comet Assay kit (Cell Biolab, San Diego, CA). 10 µL of the cell suspension and 100µL of low-melting agarose were mixed and 75ml of the mixture was immediately pipetted onto the pre-warmed OxiSelect™ Comet Slide. The slides were maintained horizontally at 4°C in the dark for 15 minutes followed by immersion in pre-chilled lysis buffer (OxiSelect™ Comet Assay kit) at 4°C for 45 minutes in the dark. The slides were than immersed in a pre-chilled alkaline solution (pH>13) for 30 minutes in the dark. Slides were later transferred to electrophoresis chamber containing alkaline electrophoresis solution, and electrophoresis was performed at 20V 300mA for 20min. After electrophoresis, the slides
were washed with water twice, and then was immediately placed in 70% ethanol for 5 min and air-dried overnight at room temperature. Cells were stained with Vista Green DNA Dye®, dried, and images were recorded using an epifluorescent microscope (Zeiss Axiovert Widefield Microscope) with FITC filter. The images were analyzed by Comet Assay IV software from Perceptive Instruments Ltd (Bury St Edmunds, UK). Data were based on 50 randomly selected cells per sample. The tail migration has been considered to be an appropriated index of induced DNA damage.

3-4. RESULTS

3-4-1. ECL fluidic chip and LC-MS/MS. The metabolic fate of chemicals and drugs in human organs depends on oxidations catalyzed by cyt P450s, as well as sequential conjugative reactions (Scheme 3-2). Cyt P450s are the major enzymes responsible for oxidative metabolism that can result in reactive metabolites and is often called bioactivation. Conjugative enzymes can catalyze reactions of primary oxidation products (Scheme 3-2) to give new products that are either bioactivated or unreactive (detoxification). Uridine 5'-diphospho-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), and epoxide hydrolases are the main enzymes involved in conjugation reactions. UGTs transfer a glucuronic acid moiety to hydroxyl, carboxyl, carbonyl and amino groups from uridine 5'-diphospho-glucuronic acid and this process generally leads to detoxification. SULTs transfer a sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate, while NATs transfer an acetyl group from acetyl Co-A to an acceptor group of the substrate. All of these enzymes from microsomes, cytosol, and single cytP450 supersomes were used in the arrays chips and on the magnetic beads for LC-MS/MS.
We chose 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 2-acetylaminofluorene (2-AAF), and styrene as test compounds for this study. NNK and 2-AAF show high rodent liver toxicity and DNA damage rates. The genotoxic influence of styrene is more moderate, but it forms known guanine and adenine adducts in DNA. NNK is one of the most prevalent carcinogens in cigarette-smoke. 2-AAF was originally developed as an insecticide, but discontinued after evidence of carcinogenicity. Styrene is widely present in the environment due to emissions from industrial processes, cigarette smoke and combustion of styrene polymers, and is well-known for moderate genotoxic effects upon metabolic activation.

**Scheme 3-2:** Simplified metabolic pathways of drugs and chemicals in humans (X) representing oxidative metabolism and conjugative metabolism to facilitate excretion, which can result in DNA adducts due reactions of nucleobases with reactive intermediates.

Knowledge of the metabolism of the parent compounds is a critical starting point for investigating the chemistry of genotoxicity. While metabolic profiles can also be developed using our biocolloid reactors and LC-MS/MS, here we utilize existing literature to establish a metabolic framework.

**3-4-2. Studies of NNK.** Our first case study involves reactions of NNK metabolites with DNA. DNA adduct measurements in lung tissues of rodents exposed to cigarette smoke as well as
human epidemiology studies ascertain tumorigenic properties of NNK.\textsuperscript{33(c)} Despite extensive studies on NNK metabolism and genotoxicity, to the best of our knowledge there are no comprehensive reports of comparative cell-free in vitro studies on the effect of specific human organs on NNK-related genotoxicity. Significant concentrations of NNK metabolites have been observed in human liver and lung.\textsuperscript{33(b)} Humans pancreatic metabolites\textsuperscript{37} were reported to generate pancreatic tumors in rats.\textsuperscript{38}

The proposed metabolic routes of NNK, a pyridine derivative, features oxidation of the pyridine ring to yield minor products such as 6-hydroxy and N-oxide derivatives of NNK and reduction of the keto group NNK to yield major intermediate, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, 1, Scheme 3-3).\textsuperscript{33} NNK and NNAL can be oxidized by cyt P450 enzymes at carbons \textit{alpha} to the nitroso group to generate methyl hydroxylated or methylene hydroxylated products (Scheme 3-3). Methylenediazohydroxide, a reactive intermediate is formed from methylenehydroxylated NNK or NNAL, which in turn, reacts with DNA to form methylated DNA adducts such as O6-Methyl-guanine (2, Scheme 3-3) and N7-Methyl-guanine (3, Scheme 3-3).\textsuperscript{33} Methyl hydroxylated NNK further rearranges to reactive species that react with guanine nucleobases to yield O6-pyridyloxybutyl-guanine (4, Scheme 3-3). Methyl hydroxylated NNAL generates O6-Pyridylhydroxybutyl-guanine (5, Scheme 3-3) upon reaction with DNA.\textsuperscript{33}
Scheme 3-3. Proposed metabolic pathways of NNK in humans.\textsuperscript{33} Numbered structures represent key intermediates and products.

Using the fluidic arrays, reactions with a representative suite of metabolic enzymes from each organ are first run simultaneously on the chip under constant reactant and cofactor feed. We selected supersomes of single cyt P450 enzymes that match the most abundant cyt P450 for each organ and are most likely to be involved in metabolism of the test compound. Cyt P450 3A4 is an abundant enzyme in human liver.\textsuperscript{39} Cyt P450 2A6, which a major oxidative enzyme in lung,\textsuperscript{9} and is heavily involved in NNK metabolism.\textsuperscript{33} Cyt P450 3A5 and cyt P450 1B1 the most abundant drug metabolizing enzymes present in human intestine and kidney respectively.\textsuperscript{9,39}
After the enzyme reactions ECL captured from array spots containing different human organ enzymes showed increases in intensity with enzyme reaction time (Figure 3-1a) This results mainly from formation of covalent adducts of metabolites with nucleobases that disorder the DNA double helix.23,24, Slopes of ECL intensity vs. enzyme reaction time (Figure 3-1b,c) are directly related to the rate of DNA damage as previous confirmed by LC-MS/MS.4,22

Relative DNA damage rates were expressed as \( \frac{mg \ of \ protein}{s \cdot mM \ NNK} \) from DNA-reactive metabolites as turnover rates for the enzyme reactions by dividing initial slopes in %ECL s\(^{-1}\) by the total amount of protein in each film and mM NNK (Figure 3-1(d), (e)). Data for liver indicate similar DNA damage rates with and without cytosolic enzymes. For the other organs, relative DNA damage rates of films containing cytosolic enzymes were significantly smaller compared to using only microsomal enzymes or single cyt P450 supersomes. This is presumably due to turning on detoxification pathways with cytosolic conjugative enzymes, thus removing a fraction of the DNA-reactive metabolites during the metabolic reactions.30 Cytosolic fractions are rich sources of major conjugative enzymes such as UGTs, and SULTs, responsible for the detoxification of reactive metabolites.30,28

Human lung tissue fractions gave the highest rate of DNA damage upon exposure to NNK under our assay conditions, suggesting that lung is the human organ most affected by NNK’s reactive metabolites (Figure 3-1(d)). Cyt P450 2A6 is revealed as the major cyt P450 responsible for
Figure 3-1. ECL array data from spots containing Ru\textsuperscript{II}PVP/enzyme/DNA films reacted with oxygenated 150 µM NNK in pH = 7.4 phosphate buffer + necessary cofactors with bioelectronic activation of cyt P450s at −0.65 V vs. Ag/AgCl (0.14 M KCl) at different reaction times. (a) Reconstructed, recolorized ECL array images. Control spots contained liver microsomes subjected to the same reaction conditions as above without exposure to NNK. Graphs on right show influence of enzyme reaction time on % ECL increase for reaction with 150 µM NNK at pH = 7.4, (b) with human organ tissue enzymes, (c) with individual cyt P450 supersomes; error bars represent SD for n = 4. Bar graphs show relative DNA damage rate (\text{\frac{mg of protein}{s}} \cdot \text{mM}^{-1}) upon exposure to NNK for (d) human organ tissue enzymes, (e) cyt P450 supersomes; color codes as in (b) and (c).
genotoxicity-related metabolic chemistry of NNK among cyt P450s tested as it demonstrates the greatest rate of DNA damage upon exposure (Figure 3-1(e)). A small rate of DNA damage was found for kidney, presumably due to the lack of NNK-metabolizing cyt P450s. Cyt P450 1B1 and 2B6 are the most prevalent enzymes in human kidney,\textsuperscript{39} but cyt P450 1B1 also gave a small rate of DNA damage, presumably due to slow metabolism of NNK. Intestine and liver demonstrated intermediate rates of DNA damage due to the presence of relatively low levels of NNK metabolizing cyt P450s such as cyt P450 2A6.\textsuperscript{9,39} Observations are generally consistent with previously findings from human in vitro microsomal assays and studies of animal models.\textsuperscript{33,40}

Amounts of individual DNA adducts generated from bioactivation of test compounds with human tissue enzymes were measured by LC-MS/MS to complement ECL array results. Magnetic beads (1 mm) decorated with the same DNA/enzyme films used in the ECL array served as biocolloid reactors in a 96-well filter plate high-throughput platform to generate DNA adducts, followed by hydrolysis of the DNA, filtration and LC-MS/MS quantitation of the individual nucleobase adducts.\textsuperscript{26} 7-Methylguanosine was used as an internal standard. Single reaction monitoring (SRM) chromatograms for the characteristic mass transition 299-152 of the O6-pyridyloxybutyl-guanine adduct (4, \textit{m/z} =299) from reaction products of NNK metabolized by human organ tissue fractions (Figure 3-2a) and reactions with cyt P450 supersomes in (Figure 3-2b) on the enzyme/DNA beads.

O6-Pyridyloxybutyl-guanine adduct (4, \textit{m/z} =299) is a depurination product under neutral thermal hydrolysis and the facile loss of pyridyloxobutyl moiety (\textit{m/z} =148) under collision-induced dissociation gives rise to the signature mass transition of \textit{m/z} 299-152.\textsuperscript{41} DNA adducts 5 (\textit{m/z} =417) and 2 (\textit{m/z} =282) were found after enzyme hydrolysis of the damaged DNA on the
bioreactor beads. SRM chromatograms for mass transition \( m/z \) 417-301 characteristic of adduct 5, \( m/z \) 282-166 for 2, and \( m/z \) 166-149 for 3 are in Figure 3-3. The sum of adducts

**Figure 3-2:** Single reaction monitoring (SRM) LC-MS chromatogram for \( m/z \) transition 299-152 monitoring formation of O6-Pyridyloxybutyl-guanine (4, Scheme 3-3) adducts from biocolloid reactors (enzyme color code on bottom) featuring (a) human organ microsomes, (b) cyt P450 supersomes; (c) and (d) are total DNA adducts found (pmol \{mg of protein\}\(^{-1}\) \{mM of NNK\}\(^{-1}\)) after reactions with 150 mM NNK at pH = 7.4 for 18 hrs, (c) human organ microsomes, (d) cyt P450 supersomes.
**Figure 3-3:** Single reaction monitoring (SRM) chromatograms for m/z transitions (a) 166-149 monitoring formation of N⁷-Methyl-guanine adducts (3, Scheme 3-3), (b) 282-166 of O⁶-Methyl-guanine adducts (2, Scheme 3-3) and (c) 417-301 of O⁶-Pyridylhydroxylbutyl-guanine (5, Scheme 3-3) from biocolloid reactors (color code link on bottom) containing human organ microsomes and cyt P450 supersomes reacted with 150 mM NNK.
2, 3, 4, 5 (Figure 3-2a,c) was largest for lung tissue enzymes followed by liver and intestine which were quite similar. Human kidney enzymes did not provide significant DNA adduct levels. The largest levels of DNA adducts were observed with cyt P450 2A6 (Figure 3-2b,d)) followed by cyt P450 3A4 and cyt P450 3A5. Cyt P450 1B1 did not provide measurable adducts.

DNA adduct amounts from LC-MS/MS are well correlated with ECL array results (Figure 3-1) giving total amounts of adducts and DNA damage rates in the order lung>liver, intestine>kidney consistent with published literature. This is a particularly relevant in that a major NNK exposure route is inhalation by tobacco smoking. In nearly all array and LC-MS/MS experiments, cytosolic enzyme decreased DNA damage or adducts consistent with significant detoxification by bioconjugation reactions. Adduct 4 (Scheme 3-3) was found at levels 5-fold or more than 2, 3 or 5 (Table 3-1), consistent with metabolic DNA damage by NNK arising mainly from initial methyl hydroxylation, followed by loss of CH₂=O to give the guanine-reactive species leading to 4 (Scheme 3-3). Thus, these results support a pathway yielding 4 is as a possible major genotoxic metabolic pathway in humans, as suggested earlier.
Table 3-1: DNA adducts (pmol \(\text{microgram of protein}}^{-1} \text{mM of NNK}}^{-1}\) formed by biocolloid reactors after reaction with 150 mM NNK at pH = 7.4 for 18 hrs.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of DNA adducts (pmol (\text{microgram of protein}}^{-1} \text{mM of NNK}}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adduct 4</td>
</tr>
<tr>
<td>HLM</td>
<td>7.12 ± 0.18</td>
</tr>
<tr>
<td>HLM+HLC</td>
<td>5.85 ± 1.00</td>
</tr>
<tr>
<td>HLMuM</td>
<td>13.07 ± 0.70</td>
</tr>
<tr>
<td>HLMuM+HLMuC</td>
<td>8.85 ± 1.44</td>
</tr>
<tr>
<td>HIM</td>
<td>7.89 ± 0.43</td>
</tr>
<tr>
<td>HIM+HIC</td>
<td>4.36 ± 0.31</td>
</tr>
<tr>
<td>HKM</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>HKM+HKC</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>cyt P450 3A4</td>
<td>111.14 ± 13.90</td>
</tr>
<tr>
<td>cyt P450 3A4+HLC</td>
<td>28.76 ± 6.87</td>
</tr>
<tr>
<td>cyt P450 2A6</td>
<td>263.13 ±15.90</td>
</tr>
<tr>
<td>cyt P450 2A6+HLMuC</td>
<td>77.51 ± 6.88</td>
</tr>
<tr>
<td>cyt P450 3A5</td>
<td>115.42 ± 8.68</td>
</tr>
<tr>
<td>cyt P450 3A5+HIC</td>
<td>68.33 ± 11.70</td>
</tr>
<tr>
<td>cyt P450 1B1</td>
<td>0.20 ± 0.11</td>
</tr>
<tr>
<td>cyt P450 1B1+HKC</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>
3-4-3. **Studies of 2-AAF.** Metabolism of 2-AAF begins with oxidation of the secondary amido nitrogen by cyt P450s to yield N-hydroxy-2-AAF (6, Scheme 3-4).[^42] Microsomal deacylases convert 2-AAF to 2-aminofluorene (2-AF) via elimination of the acetyl functional group which undergoes hydroxylation to give N-Hydroxy-2-AF (7, Scheme 3-4). Conjugative enzymes such as SULTs and UGTs further metabolize 7 to glucuronides and sulphates to facilitate clearance from the body (Scheme 3-4).[^34,42] However, glucuronide, sulphate and acetyl conjugated products can also undergo spontaneous heterolysis of the N–O bond to form arylnitrenium ion, which can react with C8 positions on guanine to form N-(Deoxygunaosin-8-yl)-2-aminofluorene (8, Scheme 3-4), N-(Deoxygunaosin-8-yl)-2-acetylaminofluorene (9).[^42]
Table 3-2: DNA adducts (pmol \{microgram of protein\}^{-1} \{mM of 2-AAF\}^{-1}) formed by biocolloid reactors after reaction with 250 mM 2-AAF at pH = 7.4 for 4 hrs.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of DNA adducts (pmol {microgram of protein}^{-1} {mM of 2-AAF}^{-1})</th>
<th>Adduct 8</th>
<th>Adduct 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>0.54 ± 0.15</td>
<td>0.15±0.17</td>
<td></td>
</tr>
<tr>
<td>HLM+HLC</td>
<td>0.22 ± 0.06</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>HLuM</td>
<td>0.39±0.04</td>
<td>0.06±0.02</td>
<td></td>
</tr>
<tr>
<td>HLuM+HLuC</td>
<td>0.49±0.09</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>HIM</td>
<td>0.42±0.07</td>
<td>0.76±0.33</td>
<td></td>
</tr>
<tr>
<td>HIM+HIC</td>
<td>0.41±0.19</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>HKM</td>
<td>0.43±0.04</td>
<td>0.39±0.03</td>
<td></td>
</tr>
<tr>
<td>HKM+HKC</td>
<td>0.18±0.07</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>cyt P450 1A2</td>
<td>4.45±0.65</td>
<td>0.23±0.16</td>
<td></td>
</tr>
<tr>
<td>cyt P450 1A2+HLC</td>
<td>0.70±0.44</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>cyt P450 2A6</td>
<td>0.82±0.35</td>
<td>0.17±0.07</td>
<td></td>
</tr>
<tr>
<td>cyt P450 2A6+HLuC</td>
<td>0.87±0.36</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>cyt P450 3A5</td>
<td>0.69±0.30</td>
<td>0.15±0.05</td>
<td></td>
</tr>
<tr>
<td>cyt P450 3A5+HIC</td>
<td>0.65±0.27</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>cyt P450 1B1</td>
<td>0.08±0.01</td>
<td>0.30±0.03</td>
<td></td>
</tr>
<tr>
<td>cyt P450 1B1+HKC</td>
<td>0.09±0.04</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Note:- N.D. Not detectable
Upon exposure to 2-AAF, microsomal enzymes gave a slight trend in DNA damage (Figure 3-4a), but differences had low statistical significance by t-tests (Table 3-3). With cytosolic enzymes included, DNA damage rates for intestine and kidney microsomes were significantly larger (Table 3-3) than for liver and lung (Table 3-3), with significantly lower rates of DNA damage in the

**Scheme 3-4.** Proposed general metabolic pathways of 2-AAF in humans yielding genotoxic and detoxified products.\(^{34,42}\) Numbered structures represent key intermediates and products.
Table 3-3: T-test values, $t_{cal}$ - Calculated t value, $t_{tab}$- Critical two-tailed t value for n=4 at 95% confidence interval = 3.182. If the value in the table below is >3.185, the two relevant sets of date are significantly different at the 95% confidence level (P=0.5). Green highlighted labels reflect data averages that are not significantly different. A: without cytosol & B: with cytosol.

<table>
<thead>
<tr>
<th></th>
<th>Styrene</th>
<th>2-AAF</th>
<th>NNK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver vs Lung</td>
<td>Liver vs Intestine</td>
<td>Liver vs Kidney</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>1.08</td>
<td>0.95</td>
<td>1.08</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>2.23</td>
<td>2.63</td>
<td>3.15</td>
</tr>
<tr>
<td>3A4 vs 2A6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>1.22</td>
<td>0.26</td>
<td>32.06</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>0.69</td>
<td>0.20</td>
<td>0.75</td>
</tr>
<tr>
<td>1A2 vs 2A6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>2.39</td>
<td>0.35</td>
<td>4.01</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>3.15</td>
<td>10.09</td>
<td>12.40</td>
</tr>
<tr>
<td>1A2 vs 3A5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>11.37</td>
<td>6.79</td>
<td>27.18</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>4.92</td>
<td>4.43</td>
<td>15.57</td>
</tr>
<tr>
<td>3A4 vs 2A6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>6.84</td>
<td>2.87</td>
<td>15.19</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>5.43</td>
<td>1.91</td>
<td>13.62</td>
</tr>
</tbody>
</table>
same trend of kidney>intestine>lung>liver. For comparison, rodent studies revealed the formation of tumors in liver>intestine>kidney upon chronic dietary exposure to 2-AAF, showing that the same organs are impacted in rodents, but not necessary in order of DNA damage from human metabolism.

We used cyt P450 1A2 supersomes for 2-AAF to replace cyt P450 3A4 since cyt P450 1A2 is the major cyt P450 responsible for metabolism in human liver. A significantly larger rate of DNA damage was found for cyt P450 1A2 (Figure 3-4(b)) compared to cyt P450s 2A6, 3A5 and 1B1, suggesting a higher rate conversion by 1A2 leading to reactive metabolites. Cyt P450 1B1 is a major cyt P450s in kidney, but DNA damage-related activity towards 2-AAF was small (Figure 3-4b), consistent with in vitro rodent mutagenicity reports.
Figure 3-4. DNA damage rates and amounts of adducts from 2-AAF. Top panel - Relative DNA damage rate (\(\text{mg of protein}^{-1} \text{s}^{-1} \text{mM}^{-1}\)) from ECL arrays exposed to 250 µM of 2-AAF in pH = 7.4 for (a) human organ tissue fractions, (b) cyt P450 supersomes. Bottom panel – Total amount of DNA adducts found (pmol \(\text{mg of protein}^{-1} \text{mM of 2-AAF}^{-1}\)) after reactions with 250 mM 2-AAF at pH = 7.4 for 4 hrs, (c) human organ microsomes, (d) cyt P450 supersomes. The ECL images and single reaction monitoring LC-MS/MS chromatograms are in Figure 3-5 and Figure 3-6.
**Figure 3-5:** ECL array data from spots containing optimized Ru^{II}PVP/enzyme/DNA film assemblies reacted with oxygenated 250 µM of 2-AAF in pH = 7.4 phosphate buffer + necessary cofactors with bioelectronic activation of cyt P450s at −0.65 V vs. Ag/AgCl (0.14 M KCl) for reaction times from 0–90 s. (a) Reconstructed and recolored ECL array images. Control spots contained liver microsomes, and were subjected to the same reaction conditions as above without exposure to 2-AAF. Influence of enzyme reaction time on % ECL increase for fluidic sensor chips reacted with 250 µM of 2-AAF in pH = 7.4, (b) with human organ tissue fractions, (c) with cyt P450 isoforms, where error bars represent standard deviations for n = 4. The relative DNA damage rate (\( \text{mg of protein}^{-1} \text{s}^{-1} \text{mM}^{-1} \)) upon exposure to 2-AAF at analytical spots containing (d) human organ tissue fractions, (e) cyt P450 supersomes.
Figure 3-6: Single reaction monitoring (SRM) chromatograms monitoring formation of DNA adducts by biocolloid reactors (color code link on bottom) after reaction with 250 mM 2-AAF at pH = 7.4 for 4 hrs; (a) m/z transition 447-331 of N-(Deoxygunaosin-8-yl)-2-aminofluorene (8, Scheme 3-4) adducts from biocolloid reactors containing human organ microsomes, (b) cyt P450 supersomes. Panels (c) and (d) show SRM chromatogram for m/z transition 489-373 monitoring formation of N-(Deoxygunaosin-8-yl)-2-acetylaminofluorene (9, Scheme 3-4) (c) human organ microsomes, (d) cyt P450 supersomes.
N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (9, m/z =489) is a major DNA adduct from 2-AAF, suggesting reaction of 6 with guanine C8 and subsequent loss of sugar (m/z =116) as collision-induced dissociation product of m/z =373. Deacetylation of 2-AAF gives 2-AF, which undergoes bioactivation to generate N-(deoxyguanosin-8-yl)-2-aminofluorene (8, m/z =447) with product ion m/z =331 (Scheme 3-4). LC-MS/MS revealed both 8 and 9 as products from their SRM chromatograms (Figure 3-6). Total levels of 8 and 9 from were highest for intestine, and cyt P450 1A2 produced large amounts of these adducts (Figure 3-4d), presumably due to faster conversion of 2-AAF. Liver and lung enzymes generated larger amounts of 8 than 9, except for intestine and kidney microsomes alone (See Table 3-2). In their presence of cytosol enzymes, 9 was not detected. Results suggest that generation of 8 may be a more significant genotoxic pathway.

3-4-4. Styrene. Studies on styrene metabolism demonstrated that cyt P450 mediated oxidation yields styrene oxide, which forms N7 guanine adducts (10, Scheme 3-5). Relative rates of DNA damage by styrene did not show statistically significant variations across different organs with or without cytosolic enzymes (Figure 3-7a, Table 3-3). This is because many cyt P450s metabolized styrene with similar efficiency. Thus, ECL arrays (Figure 3-7b) showed that cyt P450 3A4, 2A6 and 3A5 but not cyt P450 1B1 gave similar rates of DNA damage. The N7-guanine adduct of styrene oxide) (10, m/z =272) is the major DNA adduct, which results in MS/MS product ion m/z =152. In LC-MS/MS, m/z transition 272-152 (Figure 3-7c,d) revealed decreases in DNA adduct levels when including cytosolic enzymes, similar to the array results.
**Figure 3-7.** DNA damage rates and adduct amounts from styrene. Top panel - Relative DNA damage rate (\(\text{mg of protein}^{-1} \text{ s}^{-1} \text{ mM}^{-1}\)) from ECL arrays exposed to 1 mM of styrene in pH = 7.4 for (a) human organ tissue enzymes, (b) cyt P450 supersomes. Bottom panel – Total amount of DNA adducts found (pmol \(\text{mg of protein}^{-1} \text{ mM of styrene}^{-1}\)) after reactions with 1 mM of styrene at pH = 7.4 for 1 hr, (c) human organ microsomes, (d) cyt P450 supersomes. The ECL images and single reaction monitoring LC-MS chromatograms are in Figure 3-8.
Figure 3-8: Single reaction monitoring (SRM) chromatogram for m/z transition 272-152 monitoring formation of N\textsuperscript{7} styrene oxide guanine adduct (10, Scheme 3-5) from biocolloid reactors (color code link on bottom) containing (a) human organ microsomes, (b) cyt P450 supersomes.

Scheme 3-5. Bioactivation of styrene in humans, (a) general metabolic pathways of styrene yielding genotoxic and detoxified products; (b) N\textsuperscript{7} guanine adduct formed by reaction with styrene oxide.\textsuperscript{46}
3-4-5. **Comet assays.** These assays test for cell-based DNA damage from reactive chemicals.\(^{50}\) Comet assays were done using human organ cell lines treated with test compound, followed by cell lysis. DNA is stained with fluorescent dye then subjected to electrophoresis. Damaged DNA migrates further than intact DNA, resulting in a *Comet tail-like* distribution. The extent of damage is expressed as the length of tail.\(^{51}\) Mean tail length was measured as a function of incubation time with the test compound to give relative DNA damage rate (tail migration.hr\(^{-1}\) mM\(^{-1}\)) (Figure 3-9).

![Figure 3-9](image_url)  
**Figure 3-9:** Influence of reaction time on normalized tail migration (tail migration, hr\(^{-1}\)) of treated cells (Color code at the bottom) for the compounds, NNK, 2-AAF and styrene.
Figure 3-10 shows correlation plots for relative DNA damage rates from ECL arrays and Comet assays. NNK and 2-AAF gave excellent correlations with linear regression correlation coefficients of 0.959 and 0.972, respectively, confirming that ECL array results can correspond very well with cell-based DNA damage. The Comet data confirmed significant differences in extent of DNA damage in tissues from different organs, consistent with data from ECL arrays and LC-MS/MS. On the other hand, the low correlation coefficient of 0.3 for styrene (Figure 3-10) is related to lack of specificity of the different organ enzymes for styrene leading to similar DNA damage rates in the ECL assay.

**Figure 3-10.** Correlation plots of relative DNA damage rate from ECL arrays (\(\text{mg of protein}^{-1}\) s\(^{-1}\) mM\(^{-1}\)) using microsomal and cytosolic enzymes with comet assay tail migration (hr\(^{-1}\)) for test compounds on human cell lines from liver, lung, intestine and kidney.
3-5. DISCUSSION

Human organs have unique profiles of metabolic enzymes that determine genotoxicity of specific compounds generated within that organ. Results above demonstrate the power of our high-throughput assays to elucidate possible organ-specific metabolite-related genotoxic pathways and their relative importance for different chemicals utilizing a broad range of metabolic enzymes. Our new ECL fluidic array establishes relative organ-specific DNA-damage rates, and coupled with bioreactor technology for LC-MS/MS determinations can identify and quantify important nucleobase-metabolite adducts and metabolic routes involved.

Positive features of our experimental approach include rapid microfluidic array analyses of metabolite-related DNA damage rates in 64 simultaneous experiments, e.g. 16 different reactions in quadruplicate (Scheme 3-1). Once nanowells are equipped with enzyme-DNA-RuIIPVP films, array experiments require up to 90 s for enzyme reactions, 3 min. washing, and 3 min. detection, so 64 experiments can be completed in ~8 min. For LC-MS/MS analysis of nucleobase-metabolite adducts, reactions with the DNA/enzyme-coated magnetic beads in 96-well filter plates feature all 4 organ enzymes in replicate experiments run simultaneously with individual experiments representing cyt P450s alone, supersomes representing single cyt P450s, and cyt P450s plus cytosolic enzymes. After reaction, DNA is hydrolyzed off the beads, and the resulting supernatant is filtered and analyzed by LC-MS/MS. Longer times than for array studies are required, but detailed molecular information and quantitation is obtained. LC takes about 40 min per individual sample, so that future time savings could be achieved by using fast, ultrahigh pressure LC.

**Influence of Cytosolic Enzymes.** Experiments combining microsomal and cytosolic enzymes should most closely mimic human genotoxicity pathways. For NNK and 2-AAF, very
significant differences were found in relative rates of DNA damage for enzymes from the different organs, and in nearly all cases cytosolic enzymes decreased DNA damage rates (Figures 3-1 to 3-3). In all cases, total amounts of DNA adducts measured by LC-MS/MS after metabolic reactions also varied for different organ enzymes, and decreased when the cytosolic enzymes were included (Figures 3-2 & 3-3). These decreases in DNA damage measured in 2 ways are related to detoxification enabled by cytosolic bioconjugation enzymes that presumably destroy DNA reactive metabolites.$^{4,8,27,28}$ As UGTs are mainly responsible for conjugative reactions of NNK oxidative metabolites,$^{33(c)}$ glucuronides are most likely the non-reactive end products after complete metabolism. Liver microsomes contain larger amounts of membrane bound UGTs compared to extrahepatic tissues.$^{31(a)}$ Thus, the effect of added liver cytosolic UGTs is not very significant for NNK (Figures 3-1d and 3-2d). For 2-AAF, detoxification pathways were also apparent and cytosolic enzymes greatly decreased rate of DNA damage and DNA adduct levels (Figure 3-4). Intestine and kidney showed significantly higher rates of DNA damage compared to liver and lung when cytosolic enzymes were present, which is related to lower amounts of relevant SULTs in human intestine and kidney that compromises detoxification.$^{53}$ The effect of cytosolic enzymes on styrene is discussed below along with other styrene data.

For NNK and 2-AAF, strong correlations were found between cell-free DNA damage rates and human organ cell-based DNA damage when using microsomes and cytosol for the same compound (Figure 3-5). For styrene, this correlation was not found. In this respect, it is important to realize that our results cannot establish genotoxicity per se, but reveal possible chemical pathways and dynamics of genotoxic chemistry. For our examples, NNK and 2-AAF chemistry and dynamics are quite consistent with results from cell-based Comet assays. ECL array results are dominated by the enzyme chemistry leading to reactive metabolites, and cyt
P450s are somewhat non-selective and have similar activities for styrene. Small differences in the comet assay results may be influenced by effects on metabolic or DNA reaction chemistry that are not accurately mimicked in our cell-free assay. However, the comet assay is not an absolute standard for human genotoxicity, only a way to measure metabolic DNA damage in cells. Thus, the lack of ECL array-comet assay correlation for styrene does not negate the genotoxicity pathway and dynamics results found in our cell-free assays, which may still be relevant to humans.

**NNK.** The largest DNA damage rate (Figure 3-1) and highest levels of DNA adducts (Figure 3-2) for NNK were found using human lung enzymes. Considering the metabolic pathway, DNA adducts 2, 3, 4 and 5 ultimately result from a-hydroxylation of NNK and NNAL (Scheme 3-3). However, adduct 4 was found at levels 5- to 500-fold larger than 2, 3 and 5 except for kidney and cyt P450 1B1, suggesting that methyl hydroxylation leading to 4 may be the major genotoxic metabolic pathway of NNK in human lung, liver and intestine (Scheme 3-3 and Table 3-1).

Among the supersomes, cyt P450 2A6 gave the largest DNA damage rate and amounts of adducts (Figures 3-1 and 3-2) suggesting high activity for NNK oxidation to reactive metabolites. As a major metabolic enzyme in the human respiratory tract, cyt P450 2A6 is a likely candidate for genotoxic bioactivation of NNK in human lung. Consistent with these results, Smith et al. concluded a major role of cyt P450 2A6 in NNK metabolism with human lung microsomes from *in vitro* cyt P450 inhibition. This is a very relevant finding as NNK is a major component in cigarette smoke, and the lung is a major NNK target among smokers. Cyt P450 1B1 appears to be minimally involved in genotoxic pathways since a low rate of DNA damage were found (Figure 3-1d) and no DNA adducts were found (Figure 3-2d). Consistent
with cyt P450 1B1 as major human kidney enzyme, microsomal kidney enzymes showed low rates of DNA damage (Figure 3-1c) and low levels of adducts (Figure 3-2c).

2-AAF. Relative DNA damage rates for 2-AAF showed a slight decreasing trend from kidney>intestine>lung>liver when using only microsomal organ enzymes (Figure 3-4a). However, addition of cytosolic enzymes accentuates this trend. Metabolism of 2-AAF or its deacylated form 2-AF begins with oxidation of amido nitrogen by cyt P450s to yield an N-hydroxylated intermediates. (6 and 7, Scheme 3-4). In vivo studies of animal models revealed variable adduct levels based on route of administration. For example, male rats given intraperitoneal 2-AAF had higher levels of DNA adduct levels in liver than in bladder. In contrast, dietary administration of 2-AAF to mice resulted in higher DNA adduct levels in bladder than in liver. Our LC-MS/MS adduct assays revealed formation of major adducts N-(deoxyguanosin-8-yl)-2-aminofluorene, (8) and N-(deoxyguanosin-8-yl)-2-acetylaminofluorene, (9) which are the most commonly reported adducts in cell-based in vitro assays and animal tests. The C8 position is less sterically hindered compared to other nucleophilic sites of guanine and reacts readily with bulky 2-AAF metabolites. When the full complement of cyt P450s and cytosolic enzymes were used, no 9 was found. This suggests that the route from 7 to 9 is not an important genotoxic pathway (Scheme 3-4), and routes to 8 via 6 and 7 predominate. The largest DNA damage rate and adduct levels for 2-AAF was found with supersomal cyt P450 1A2 (Figures 3-6b & 3-6d), consistent with reported bioassays.

Styrene. Rates of DNA damage by styrene metabolites did not differ at the 95% confidence level with type of organ tissue enzymes (Figure 3-7). ECL array results are consistent with broad enzyme specificity for styrene since cyt P450s 3A4, 2A6 and 3A5 show similar DNA damage rates, although cyt P450 1B1 had low activity (Figure 3-7). Inclusion of cytosolic
enzymes gave very large decreases in DNA damage rates and amount of adducts (Figure 3-7) for all organs except lung. This is significant since lung inhalation is an exposure route for styrene.\(^{59}\) In general, results are related to efficient detoxification of styrene oxide to phenylethylene glycol by cytosolic epoxyl hydrolase (EH).\(^ {46,48}\) Rate of DNA damage halved upon using microsomes and cytosol (Figure 3-8(a)), but decreased 10-fold when using cyt P450 supersomes and cytosol (Figure 3-8(b)). This is because organ microsomal fractions contain significant membrane bound EH but supersomes do not. Thus, human microsomes without cytosols styrene oxide is hydrolyzed to phenylethylene glycol, lowering DNA damage rates.\(^ {60}\) Glucuronide metabolites have been found in rodents, consistent with a metabolic route in Scheme 6.\(^ {61}\)

### 3-6. CONCLUSION

High-throughput microfluidic array and magnetic bead reactor-LC-MS/MS strategies enabled the use of organ-specific suites of metabolic enzymes to establish rates and pathways of metabolite-related DNA damage. These studies identified nucleobase adducts from DNA damage by reactive metabolites, pinpointed enzymes that form the reactive metabolites, established relative dynamics of enzymes suites in the different organs, and helped reveal pathways of possible genotoxic chemistry. Results illustrate the power of combining DNA/enzyme ECL microarray/LC-MS/MS to comprehensively and efficiently explore organ-specific metabolic genotoxic pathways related to the drugs and environmental chemicals in the human body.
3-7. REFERENCES


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Chapter 4

Covalent Protein Modification by Reactive Drug Metabolites using Magnetic Biocolloid Reactors and Liquid chromatography/ Mass spectrometry

4-1. ABSTRACT

Covalent binding of reactive metabolites to proteins is considered to be one of the major causes for adverse drug reactions. Consequently, there is a great need in early stage drug candidate screening methodologies that feature in vitro generation and characterization of reactive metabolites induced protein adducts. We have developed a simple and label-free drug-protein adducts evaluation method by using magnetic bead technology involving layer-by-layer coating with polyelectrolytes and metabolic enzymes as biocolloid reactors, and coupling with capillary liquid chromatography-tandem mass spectrometry. This technique was illustrated by the bioactivation of acetaminophen (APAP) into its reactive metabolites, N-acetyl-p-benzoquinoneimine (NAPQI), which covalently binds to the model target protein, human glutathione S-transferase pi (hGSTP), in the reaction buffer. The modified/unmodified hGSTP is then digested and characterized by capillary liquid chromatography/tandem mass spectrometry. This method successfully enabled detection of NAPQI-modified peptides 45-54 and characterization of the adduct structure for hGSTP at Cys-47. We were able to separate metabolic enzymes from targeted proteins, and effectively generate and characterize the reactive
metabolites induced protein adducts. Therefore, this method may provide a useful tool to predict the potential covalent protein binding for drug candidates.
4-2. INTRODUCTION

It has been almost 70 years since Elizabeth and James Miller first discovered the covalent binding between a methylaminoazobenzene carcinogen and tissue proteins.\(^1\) In the early 1970s, drug-protein adduct induced toxicity was first analyzed by radiolabeled hepatic toxicant covalent binding to liver proteins in rat and mice.\(^2,3\) There are two major problems for comprehensive characterization of drug-protein adducts. First, covalent binding level generally is relatively low such that differentiating the small portion of adducted protein from complex cell proteomes is often known as a “needle in a haystack” problem. Second, the potential target proteins are highly diverse and complicated which increase the analysis difficulty. Until recently, many efforts have been put into the covalent modification of proteins by xenobiotics. Most drug-protein adduct studies are still based on radiolabeled compounds bound to bulk tissue or cell proteins, and followed by various separation and targeted protein enrichment strategies:\(^4-6\) Immunochemical detection was based on antibodies against target proteins using Western blotting and Edman sequencing.\(^7\) But this approach lacks efficient adduct enrichment and the result actually indicates only immunoreactive proteins not specific adducted proteins. Qiu et al. employed 2D SDS-PAGE coupled with matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) to identify 23 acetaminophen metabolites which caused damage in hepatic proteins.\(^8\) This method provided higher separation resolution and identification reliability, which appeared as the first universal method for protein covalent modification discovery.\(^9\) Another strategy applied biotin labeling on adducted proteins for affinity capture by shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.\(^10\)

Radiolabeling is a sensitive method for the identification of drug-protein adducts. However, it is normally only performed on late-stage preclinical candidates due to its high cost.
Consequently, a simple methodology for comprehensive generation and evaluation of drug-protein covalent binding with high sensitivity and low cost is highly desirable. We recently develop a label-free and inexpensive assay that can enzymatically generate drug-protein adducts using magnetic biocolloid reactor. Magnetic biocolloid reactor particles are fabricated based on electrostatic layer-by-layer (LbL) self-assembly method. Polyelectrolytes and metabolic enzymes with opposite charges are alternately deposited on carboxylate-functionalized negatively charged magnetic particles driven by coulomb interaction. Multiple layers of electrolytes provide continuous surface charge and large surface area for enzyme film growth. Enzymes are immobilized onto the polyelectrolytes-coated surface without losing catalytic activity. The coated particles provide a magnetic property that allows simple and rapid separation by a permanent magnet, which potentially can be further developed for high throughput screening.

Acetaminophen (APAP) has been well-known as an analgesic and antipyretic agent for over a hundred years. It is also responsible for 80% of drug-related liver failure cases. Hepatotoxicity study of APAP was initiated by Jollow et al. in 1973. They found that APAP can be bioactivated by microsomal enzymes and transformed into reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). NAPQI can be efficiently detoxified by reduced glutathione under therapeutic doses. Glucuronidation and sulphonation of APAP are the other two major metabolic pathways that can deactivate APAP for rapid excretion in urine. In case of acute overdose, glutathione depletion results in saturation of liver detoxifying capacity. NAPQI covalently binds to microsomal proteins, which can lead to centrilobular hepatic necrosis (Scheme 1). Liver microsomal glutathione S-transferase (GST) is an abundant enzyme that can catalyze the conjugation of reduced glutathione (GSH). With depletion of GSH content in
the liver, GST becomes one of the preferable covalent binding biomolecules for NAPQI. In this context, a member of GST family, human glutathione S-transferase pi (hGSTP) is used as a model target protein for APAP hepatotoxicity study. Because it can be easily expressed in E. coli and has been well characterized. It is a relatively small protein that consists of four cysteine residues (Cys-14, 47, 101 and 169), among which Cys-47 has the highest reactivity due to its relatively low pKa (3.5-4.2) and accessibility. Several studies reported that covalent modification of Cys-47 can lead to significant reduction in hGSTP enzyme activity.

Herein, we report a label-free in vitro assay using enzyme/polyelectrolytes-coated magnetic biocolloid reactors for APAP-hGSTP adducts generation coupled with liquid chromatography-tandem mass spectrometry for protein covalent modification structural analysis.

**Scheme 1.** Major metabolic pathways of APAP.
4-3. EXPERIMENTAL SECTION

4-3-1. Chemicals and materials

Human liver supersomes 2E1 (Cyt P450 2E1OR with p450 reductase and Cytochrome b5) were purchased from CORNING (Woburn, MA, USA). Carboxylated magnetic particles were from Polysciences (Warrington, PA; 1 µm diameter; concentration 20 mg mL\(^{-1}\)). Acetaminophen, Authentic N-acetyl-p-benzoquinone imine (NAPQI), urea, reduced glutathione (GSH), Glutathione S-transferase from human placenta, NADPH regenerating system: glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), and nicotinamide adenine dinucleotide phosphate (NADP), were from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), iodoacetamide (IAA) were obtained from Thermo Scientific (Rockford, IL, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). All of the solvents were of high-performance liquid chromatography (HPLC) grade from Sigma-Aldrich (St. Louis, MO, USA).

4-3-2. Film fabrication on magnetic particles

Bioreactor films were prepared according to a previous report with slight modification.\(^{27}\) 200µl of negatively charged magnetic particles (2mg ml\(^{-1}\) in tris buffer, pH 7.4) was mixed with 200µl of polycation poly(diallyldimethylammoniumchloride) (PDDA) (2mg/ml in water, containing 50 mM NaCl). The mixture was kept in ice for 20-min until PDDA adsorption saturated on the particles. Then the PDDA-coated particles were first separated from the solution using magnets, followed by washing and redispersing in 10 mM tris buffer (pH 7.4). The same assembly and washing steps were repeated for polyanion poly(styrene sulfonate) (PSS) and CYP2E1 supersomes film growth. Supersomes required 30 min to reach steady state adsorption.\(^{17}\) The final architecture on the magnetic bioreactors was MP/PDDA/PSS/PDDA/2E1.
4-3-3. Protocols for magnetic biocolloid reactors incubation

After film fabrication, magnetic biocolloid reactor particles were dispersed in 400 µl 0.1 M phosphate buffer (pH 7.4) containing NADPH-regenerating system (10 mM glucose-6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 10 mM MgCl$_2$, 0.80 mM β-NADP$^+$), 2mM DTT and 1mM APAP. For metabolites study, 1mM GSH was added to the solution to trap the reactive metabolites NAPQI. For protein adducts study, 50 µg hGSTP was used as the target protein for covalent modification of APAP metabolites. Negative control experiments were conducted in the absence of NADPH-regenerating system or APAP substrate. After incubation for 30 min at 37°C, the reaction was terminated by magnetic separation. Positive control experiment was performed by using 100 µM authentic NAPQI directly react with 50 µg hGSTP in phosphate buffer solution.

4-3-4. Tryptic digestion of hGSTP

Buffer exchange. After magnetic separation the supernatant was transferred to Amicon® Ultra centrifugal filters (Millipore, Billerica, MA, USA) with 3,000 molecular weight cutoff to desalt and exchange buffer. After loading sample solution to inner tube with mass cutoff filtering membrane, it was centrifuged at 16,000×g for 30 min to remove majority of phosphate buffer into the outer tube. Nevertheless, about 40 µl of protein containing buffer stay in the inner tube. Then 500 µl of 50 mM ammonium bicarbonate buffer (pH 8.0) was added into the inner tube, and centrifuge again for 30 min at the same speed to filter out the rest of phosphate salt. The step for removing phosphate salt was repeated twice. Finally, the concentrated sample was reconstituted with 100 µl ammonium bicarbonate buffer and subjected to tryptic digestion.

Tryptic digestion. hGSTP reduction was carried out by adding 4 µl of 500 mM DTT to the sample solution for one hour at room temperature. The free thiol group on cysteine residues were
then alkylated by addition of 8 µl of 500 mM IAA and incubated in dark for one hour at room temperature. Excess IAA was then neutralized by 8 µl of DTT for 15 min. The resulting samples were digested by sequencing grade modified trypsin in a trypsin/protein (w/w) ratio of 1:25 for 15 hours. The reaction was stopped by adding 2 µl of formic acid.

4-3-5. LC-MS/MS analysis

*NAPQI-GSH metabolites analysis.* A capillary LC (Waters, Capillary LC-XE, Milford, MA) was used as previously described.\(^\text{17,33}\) 10 µL of sample was injected to a Luna C18 trap column (0.5mm × 20mm, 5µm, Phenomenex) and flushed at a flow rate of 10 µL min\(^{-1}\) with water (with 0.1% formic acid) to eliminate the phosphate buffer salt. After 3 min, the analytes were flushed to the analytical column, Luna C18 column (0.5mm × 150mm, 5µm, Phenomenex) with A, water (with 0.1% formic acid) and B, acetonitrile (with 0.1% formic acid). The gradient was 5% B for 5 min, 5-90% B for 20 min, 90% B for 2 min, 90-5% B for 2 min, 5% B for 1 min. A 4000 QTRAP® (AB Sciex, Foster City, CA) mass spectrometer with Analyst 1.5 software was operated in the positive mode with a Turbo IonSpray ionization source. Multiple reactions monitoring (MRM) was conducted at 5300 V ion spray voltage, 275 °C, 50 V declustering potential, 30 eV collision energy, and 0.15 s dwell time for different mass transitions: internal standard (136→94), NAPQI-GSH metabolites (457→328). Enhanced product ion scanning (EPI) was performed using collision-induced dissociation (CID) with 30 eV collision energy for NAPQI-GSH parent ion m/z 457.

*NAPQI-hGSTP analysis.* 2 µL of tryptic peptide mixtures were loaded to a Jupiter C18 column (0.5 mm × 150mm, 5µm, Phenomenex). The gradient for the analytes separation in the analytical column is: 5% B for 5 min, 5-50% B for 65 min; 50-95% B for 5 min, 95% B for 5min, 95-5% B
for 3 min and 5% B for 2 min at a flow rate of 15 µL min⁻¹. Information-dependent acquisition (IDA) was performed on a QSTAR® Elite mass spectrometer (AB Sciex, Foster City, CA) equipped with a TurboIonSpray source in positive mode with a spray voltage of 5500 V and desolvation temperature of 300 °C. The method ran a full ion scan survey in the m/z range 300 to 1500, followed by MS/MS acquisition on the two most prominent precursor ions from the survey scan. After an ion was selected once it would be excluded from MS/MS acquisition in the following 30 seconds. The mass-to-charge value and retention time from negative control sample IDA scan was listed in a rejected mass list for the analysis of enzymatic incubation samples.³⁸

4-3-6. Mascot search

The LC–MS/MS data were submitted to a local mascot server for an MS/MS protein identification search using Mascot Daemon (version 2.3) against NCBInr database with the following parameter settings, Taxonomy: Homo sapiens; Fixed modifications: none; Variable modifications: oxidized methionine (M), NAPQI (C); Enzyme: trypsin; Number of allowed missed cleavages: 2; Peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.5 Da. Charge state: 2, 3 and 4. The NAPQI modified hGSTP was identified with peptides that exhibit ion score > 30 (P < 0.05).

4-4. RESULTS

4-4-1. Characterization of films on magnetic bioreactors

The total amount of enzyme incorporated in the magnetic bioreactors was estimated using Bradford assay.³⁴ (Table 1) The film composition has been modified from a previous report.³³
Triple layers of polyelectrolytes (PDDA/PSS/PDDA) were deposited onto the 1µm magnetic particle surface before metabolic enzymes adsorption. Magnetic particles modified with triple-layer composites was observed about 15% increase in 2E1 adsorption comparing to magnetic particles with single polyion layers. This indicates that multiple layers of polyelectrolytes may provide larger continuous charged surface for enzyme adsorption than single layer’s.

**Table 4-1.** Estimated Amount of Metabolic Enzymes Deposited on the Magnetic Particles

<table>
<thead>
<tr>
<th>Composition (per mg particles)</th>
<th>Metabolic enzymes (µg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDDA/2E1</td>
<td>487 ± 11</td>
</tr>
<tr>
<td>PDDA/PSS/PDDA/2E1</td>
<td>558 ± 45</td>
</tr>
</tbody>
</table>

Zeta potential was conducted to determine the Electrophoretic mobility of each surface layer in the colloidal system (Figure 1). Unmodified magnetic particles exhibited a zeta potential of -26.57 mV due to the presence of surface carboxylate ions. Surface charge sign changed with each successive polymer layer. The zeta potential of the final MP/PDDA/PSS/PDDA/2E1 bioconjugate particles appeared negatively charged. Meanwhile, the isoelectric point of hGSTP is around 4.6 which appear overall negative charge in the pH 7.4 phosphate buffer. Therefore, hGSTP was unlikely to be adsorbed to the biocolloid reactor particles due to the charge repulsion.
Figure 4-1. Zeta potential of magnetic particles modified with polyelectrolytes layers. (A) MP, (B) MP/PDDA, (C) MP/PDDA/PSS, (D) MP/PDDA/PSS/PDDA, (E) MP/PDDA/PSS/PDDA/2E1

4-4-2. Detection of NAPQI-GSH metabolites

Recombinant human CYP enzyme CYP2E1 is considered one of the major P450 isoenzymes responsible for the bioactivation of APAP.\textsuperscript{36,37} CYP2E1 was deposited on magnetic biocolloid reactor particles to initiate metabolic activation reaction of APAP and APAP was oxidized into NAPQI (Scheme 1). GSH in the incubation system was used for trapping the reactive and unstable metabolite, NAPQI. Analysis of NAPQI-GSH conjugates formation provides an indirect way to assess bioactivation of APAP.\textsuperscript{38-40} The EPI spectra of NAPQI-GSH showed typical fragmented ion patterns $m/z$ 382, $m/z$ 328, $m/z$ 311, $m/z$ 208, $m/z$ 182, $m/z$ 140, which matched well with the literature values.\textsuperscript{38,40} (Figure 2A) In the analysis, MRM was employed as semiquantitative method to evaluate the production of NAPQI-GSH conjugates. Amount of
NAPQI formation was estimated by comparing the peak area ratio with internal standard acetanilide (136→94). After 30 min incubation, the formation rate of NAPQI-GSH was found to be 0.34 nmol min\(^{-1}\) (nmol CYP2E1\(^{-1}\)) (mM APAP\(^{-1}\)). These results suggest that the CYP2E1 on magnetic biocolloid reactor particles was enzymatically active and able to convert APAP into reactive metabolite NAPQI.

**Figure 2.** LC-MS/MS results of NAPQI-GSH metabolites. (A) EPI spectrum of NAPQI-GSH; (B) TIC (Total Ion Current) chromatogram of LC-MS/MS with MRM mass transition pair \(m/z\) 457 to \(m/z\) 328.
4-4-3. Detection of NAPQI-hGSTP adducts

Due to the low levels of covalent binding between NAPQI and hGSTP in the enzyme reaction, the unreacted hGSTP was alkylated at the free thiol group of cysteine residues by IAA during tryptic digestion process. The purpose of alkylation is to protect the free thiol group from undesirable reactions which may lead to NAPQI-modified peptides detection failure. The protein sequence coverage was 71%, only considering the peptides with ion scores greater than 30 through MASCOT search. NAPQI with monoisotopic mass of 149.0476 Da was considered in the database search. As a result, NAPQI-modified peptide ASCLYGQLPK at protein position 45 to 54 was successfully identified. (Figure 3A) The m/z of the observed peptide 45-54 parent ion [M+2H]^{2+} was 614.8002 which matches with the theoretical value 614.8052. The mass accuracy was 8.72 ppm and the ion score was 51. In the negative control experiment, no modified peptide 45-54 with m/z 614.8 was found. The MS/MS spectrum of modified peptide 45-54 was consistent with the theoretical value. (Figure 3B) The modified residue was determined to be Cys-47 since the fragment ion of b_{3}^{1+} m/z 411.1561 and y_{8}^{1+} m/z 1070.5303 showed a mass shift of 149.0 Da comparing to unmodified hGSTP peptides. Meanwhile, all of the detected fragment ions that including Cys-47 residues, b ions from b_{3} to b_{8} and y ions from y_{8} to y_{9}, indicated the addition of 149.0 Da from NAPQI. No other cysteine-containing peptides were found to be modified by NAPQI.
4-5. DISCUSSION

In the present study we demonstrated a novel protein adduct generation and characterization assay using magnetic biocolloid reactor particles and LC-MS/MS analysis. The model drug APAP was activated by the CYP2E1 on the biocolloid reactor particle surface, and the resulting reactive metabolites NAPQI subsequently bound to target protein hGSTP in the reaction buffer. Electrostatic based layer-by-layer self-assembly is a simple and universal technique to immobilize charged biomolecules onto a solid charged surface, which may increase the reaction...
efficiency and simplify purification process. Iron oxide based magnetic particles provide a solid support for the films and even more shorten the separation and purification steps by magnetic power, comparing with non-magnetic material based particles. We optimized the architecture of biocolloid reactor particles by adding two more layers of polyelectrolytes before enzyme adsorption. Bradford assay results indicated that the new architecture with PDDA/PSS/PDDA/2E1 was able to adsorb about 15% more CYP2E1 enzyme than the previous architecture PDDA/2E1. The possible reasons can be: 1) Nonlinear ‘island-like’ growth at the first couple layers so that only small amount of charged macromolecules can be adsorbed; 2) The outer layers with linear growth provide continuous charge to help the enzyme adsorption; 3) Biocolloidal particles that suspended in buffer solution is able to expose effective surface area to adsorb enzymes. The magnetic separation procedure helped to terminate the reaction, as well as remove the metabolic enzyme CYP2E1 from target modified protein hGSTP. This magnetic biocolloid reactor assay provided a label-free and simple way to generate and purify reactive metabolite-protein conjugates. Recently, some other label-free protein adduct generation and characterization methods have been developed. Lohmann et al. was using electrochemical flow-through cell to generate large amount of metabolites for protein adduct production and coupled with LCMS detection. Nevertheless, it cannot mimic the biological environment that its result may be biased towards the enzymatic reaction products. Yukinaga et al. employed a fractionation procedure to isolate target modified protein hGSTP from human liver microsomes by UPLC, and followed by LC-MS/MS analysis with a reject mass list. Although this approach made a significant progress in label-free drug protein adduct detection, HPLC fractionation was time-consuming and microsomal enzymes may not completely separate from target modified proteins. In addition, bioengineered cytochrome P450 BM3 mutant (CYP102A1M11H) system
was used for bioactivation of several drugs for protein adduct generation.\textsuperscript{44,45} But this technique may only apply to P450 BM3 mutant relevant drug studies. Our present method successfully enzymatically generates NAPQI-modified hGSTP adducts by using magnetic biocolloid reactor particles and characterizes them with structural detail information by LC-MS/MS analysis.

In this study we used a capillary HPLC system coupled with a QTOF mass spectrometer to acquire LC-MS/MS data for protein sequencing and modified peptides analysis. In the method, each cycle of information dependent acquisition (IDA) consists of a full survey scan followed by two MS/MS fragmentation experiments on the two most dominant precursor ions from the previous scan. However, the covalent binding between drug metabolites and proteins is typically so low that the adducted peptide ions are usually not prominent enough to be selected for MS/MS analysis. Therefore, we used a negative control sample to generate a reject ion mass list for the IDA of bioactivation analytes sample.\textsuperscript{28} This approach could exclude the most abundant unwanted ions and give higher opportunity to select the target modified peptide ions for MS/MS. In the MASCOT database searches the mass shift caused by NAPQI modification was used as a variable modification so that the metabolite-modified peptides could be automatically recognized and sorted out. LC-MS/MS analysis presented relatively high sequence coverage to 71\% and successfully identified the modification site at Cys-47 residue. This result confirmed Cys-47 of hGSTP could be a highly reactive site for electrophiles.\textsuperscript{28} These LC-MS/MS strategies can be further developed for other drug metabolites covalent binding study.

Covalent modification of protein is very important towards drug safety assessment. Because modified protein conjugates may reduce the protein activity,\textsuperscript{46,47} affect protein-protein interaction,\textsuperscript{48} or induce protective response from element-mediated gene transcription,\textsuperscript{49} which could lead to detrimental biological consequence. However, not all covalent binding leads to
toxicity. Some non-hepatotoxic drugs may show a higher covalent binding level than hepatotoxins.\textsuperscript{5} The relationship between covalent binding and hepatotoxicity is not cleared yet.\textsuperscript{4,5} Many other factors may be involved in the toxicity metabolism of a drug, such as competing detoxification pathway and metabolic turnover rate, etc. Therefore, the interpretation of covalent binding data is complicated and prudent.

In summary, we presented a novel protein adduct generation and characterization assay using supersomes assembled magnetic biocolloid reactor particles and LC-MS/MS analysis. This approach provides a label-free, informative and sensitive \textit{in vitro} methodology, which can be applied for various drug metabolites covalent modification of protein screening at the early stage of drug development. Future work may involve using human liver microsomes as the bioactivation enzymes to achieve more comprehensive information for drug-protein adduct formation.
4-6. REFERENCE


