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Addressing Public Health Risks of Persistent Pollutants Through Nutritional Modulation and Biomimetic Nanocomposite Remediation Platforms

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ADDRESSING PUBLIC HEALTH RISKS OF PERSISTENT POLLUTANTS THROUGH NUTRITIONAL MODULATION AND BIOMIMETIC NANOCOMPOSITE REMEDIATION PLATFORMS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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Lexington, Kentucky

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Lexington, Kentucky
2014

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ABSTRACT OF DISSERTATION

ADDRESSING PUBLIC HEALTH RISKS OF PERSISTENT POLLUTANTS THROUGH NUTRITIONAL MODULATION AND BIOMIMETIC NANOCOMPOSITE REMEDIATION PLATFORMS

Due to their relative chemical stability and ubiquity in the environment, chlorinated organic contaminants such as polychlorinated biphenyls (PCBs) pose significant health risks and enduring remediation challenges. Engineered nanoparticles (NPs) provide a novel platform for sensing/remediation of these toxicants, in addition to the growing use of NPs in many industrial and biomedical applications, but there remains concern for their potential long-term health effects. Research highlighted herein also represents a transdisciplinary approach to address human health challenges associated with exposure to PCBs and NPs. The objectives of this dissertation research are two-fold, 1) to develop effective methods for capture/sensing and remediation of environmental toxicants, and 2) to better understand associated risks and to elucidate relevant protective mechanisms, such as lifestyle-related modulators of environmental disease.

Prevalent engineered nanoparticles, including aluminum oxide and titanium dioxide, have been studied to better understand effective nanoparticle dispersion methods for in vitro nanotoxicology studies. This work has served both to effectively stabilize these nanoparticles under physiological conditions and to better understand the associated mechanisms of toxicity, which links these metal nanoparticles to endothelial oxidative stress and inflammation through phosphorylation of key cellular signaling molecules and increased DNA binding of pro-inflammatory NFκB. Surface functionalization, though, is being found to limit potential toxicity and has been utilized in subsequent research.

A novel polyphenol-functionalized, NP-based system has been developed which combines the biomimetic binding capabilities of nutrient polyphenols with the separation and heating capabilities of superparamagnetic iron oxide NPs for the capture/sensing of organic contaminants in polluted water sources. Magnetic nanocomposite microparticles (MNMs) incorporating the fluorescent polyphenols quercetin and curcumin exhibit high affinity for model organic pollutants followed by rapid magnetic separation, addressing the need for sustainable pollutant remediation.
Further work has been performed to both better understand health concerns associated with environmental toxicants such as PCBs and to determine effective methods for modulating their toxicity. This research has shown that PCB remediation through dechlorination is a viable technique for decreasing endothelial inflammation, although complete dechlorination to biphenyl is necessary to effectively eliminate superoxide production, NFκB activation, and induction of inflammatory markers. Additionally, the nutrient polyphenol EGCG, found in green tea, has been shown to serve as a biomedical modulator of in vivo PCB toxicity by up-regulating a battery of antioxidant enzymes transcriptionally controlled by AhR and Nrf2 proteins.

KEYWORDS: POP toxicity; polyphenol; oxidative stress; antioxidant response; sustainable remediation
ADDRESSING PUBLIC HEALTH RISKS OF PERSISTENT POLLUTANTS THROUGH NUTRITIONAL MODULATION AND BIOMIMETIC NANOCOMPOSITE REMEDIATION PLATFORMS

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April 23, 2014
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Chapter 1. Introduction

1.1 Background

1.1.1 Combining environmental science and biomedical strategies to better protect against pollutant toxicity

Contaminant remediation and biological modulation of environmentally persistent pollutants are two crucial means of reducing the human health risks of Superfund chemicals/toxicants and related hazardous materials. The Superfund program, designed to clean up sites contaminated with a variety of hazardous substances, was formed as the United States’ response to this growing environmental and public health concern\(^1\), but these pollutants are of worldwide concern. Although complete remediation of a hazardous site may continue to be an ultimate goal of human exposure risk reduction, it is important to design and implement other biologically relevant means of buffering against toxicant exposure before, during, and after remediation activities. Of additional emerging concern, nanotechnology products, such as metal oxide nanoparticles (NPs), show much potential both for drastically improving biomedical and consumer products and as the basis for effective pollutant remediation strategies, but they also present potential physiological and toxicological concerns associated with their ability to pass through cell membranes and into the cytosol of cells\(^2\). There is a clear need to develop the potential uses of NPs in biomedical treatments and remediation platforms while concomitantly understanding potential public health concerns that may arise from their increased use.

Additionally, new data now implicate the importance of an individual’s nutritional status and the use of protective bioactive food components to decrease the overall toxicity of environmental pollutants to biological systems\(^3\). Nutrition is being substantiated as an important modulator of inflammatory and antioxidant pathways, especially with regard to environmental insults. Interestingly, novel research now shows that bioactive food components that are environmentally friendly can also be integrated into remediation technologies, which in turn allow for more sustainable, inexpensive, and effective pollutant removal and detoxification\(^4\). Implementing bioactive molecules in both biomedical and environmental science settings, together, will allow for decreased overall body burden and human toxicity of a multitude of pollutants (see Figure 1.1 for overview). Herein, the relevant literature concerning this novel paradigm is discussed,
which points to the possible future uses of bioactive food components in healthful nutrition as well as their emerging uses in environmentally friendly remediation strategies for ultimately reducing human risks to persistent environmental pollutants.

1.1.2 Exposure to POPs, risks for non-communicable diseases, and mechanisms of toxicity

Persistent organic pollutants (POPs) consist of a long list of toxic chemicals designated as persistent due to their high chemical stability. POPs have been widely banned due to their unforeseen adverse health effects\(^5\). Of special interest herein, polychlorinated biphenyls (PCBs) are a diverse class of manmade POPs that have been outlawed in the United States since the 1970s, but still pose toxicological risks due to their persistence to environmental degradation\(^6\). Marketed for their effective thermal and electrical properties, PCBs became commonplace in capacitors, caulking, and hydraulic fluids but have become notorious for their correlation to multiple human toxicities ranging from endocrine disruption to vascular inflammation\(^7\). Although PCBs are detrimental to multiple target organ systems and tissue types\(^8\), special focus of this research has been placed on their role in vascular related toxicity. Of the 209 individual congeners that were manufactured, dioxin-like coplanar PCBs such as PCB 77 and PCB 126, which lack chlorine substitutions on \textit{ortho} positions of both phenyl rings, are most toxic to the endothelium and associated vasculature\(^9\).

Haloge\(\text{n}\)nated POPs, including polychlorinated biphenyls (PCBs) and trichloroethylene, are highly prevalent in the environment in soil, air, and water\(^3\). For example, a major source of human exposure to PCBs is through dietary intake of contaminated foods and through inhalation of airborne pollutants\(^8\). Because most halogenated POPs, including PCBs, are lipid soluble, they easily accumulate in human tissues, leading to a perpetually increasing disease risk throughout a life span, especially in overweight populations\(^10\). Historically, epidemiological studies have been focused on occupational exposures such as those that occurred with Swedish capacitor workers in the mid 20\(^{th}\) century, but it is becoming clearer that chronic low-dose exposure may pose the most risk for the general public\(^11;\)\(^12\). For example, populations most at risk for chronic exposure are those that ingest high levels of fatty fish due to the fact that PCBs preferentially bioaccumulate in adipose tissue and thus can be transported vertically through the food chain\(^13;\)\(^14\). Although Inuit populations have been observed to have 3.4-
fold higher plasma PCB levels than Caucasians, low ppb concentrations in plasma are common for the general United States population\textsuperscript{15}. Importantly, according to the National Health and Nutrition Examination Survey (NHANES) there appears to be an association with plasma levels of PCBs and cardiovascular disease in women in the United States\textsuperscript{16}. Although these association studies are far from causative and can only correlate levels of PCBs with disease outcome, there are human studies that show PCB exposure can alter blood lipid profiles and increase total blood cholesterol and triglycerides, lending merit to the paradigm that environmental toxicants can promote or exacerbate vascular pathologies in humans\textsuperscript{13}.

Mechanistically, an increase in cellular oxidative stress often precedes an inflammatory response\textsuperscript{17}. PCBs, and in particular coplanar PCBs, have been shown to cause oxidative stress primarily through a cytochrome P450 (CYP1A1)-mediated uncoupling mechanism\textsuperscript{18}. CYP1A1 induction allows for the detoxification of multiple xenobiotics, but when in the presence of PCB, can become inefficient and leaky (i.e., uncoupled) and produce detrimental reactive oxygen species\textsuperscript{18}. A hallmark of the pathology of vascular diseases, including atherosclerosis, includes a change in the cellular redox status and a resultant increase in oxidative stress, which favors chronic and low level inflammation\textsuperscript{19}. Such changes in redox status and oxidative stress levels can be driven in part by pro-oxidative and pro-inflammatory environmental pollutants that are persistent and which can be easily stored in adipose tissue. Thus, an increased body burden of such persistent environmental pollutants is a particular risk factor during obesity, a disease characterized by excessive adipose tissue\textsuperscript{10}. There is sufficient evidence that several POPs contribute to inflammation by activating oxidative stress-sensitive transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)\textsuperscript{20}. For example, our studies suggest that PCBs, and in particular coplanar PCBs, can increase cellular oxidative stress and induce inflammatory parameters such as inflammatory cytokines, chemokines, and adhesion molecules in the vascular endothelium, which are metabolic events that foster an inflammatory response and atherosclerosis\textsuperscript{20-23}. Through these pro-inflammatory mechanisms, PCBs and related environmental toxicants have been correlated with increased risk of multiple human chronic disease phenotypes including diabetes and heart disease\textsuperscript{13; 24-27}. The pathology of many inflammatory diseases, especially of non-communicable or chronic diseases, develops over a long period of time and thus can be easily modulated by environmental exposures, specifically to persistent organic pollutants. Since many
populations susceptible to toxicant-induced disease are often also afflicted by diet-induced diseases, future human studies and integrated risk assessments are needed to better investigate the interaction between nutrition and toxicology\textsuperscript{28}.

1.1.3 *Potential opportunities and risks associated with nanotechnology*

Nanotechnology and the production of nano-sized particles have emerged as promising areas of study due to their many applications in industry and medicine\textsuperscript{29; 30}. In fact, it is conservatively estimated that $10 billion of industrial and governmental funding is directed toward nanotechnology research each year, and that the global production of metal oxide NPs alone, without considering engineered nanoparticles, will increase 600% over the next 5 years to 1.6 million tons\textsuperscript{31}. Nanoscale materials have several properties that cause them to behave differently than their bulk counterparts upon interacting with chemical and biological compounds, such as their size, shape, surface chemistry, presence of surface lattice defects, curvature of the NP surface, and the atomic arrangement of the crystalline facets\textsuperscript{32}. Additionally, nanomaterials have increased surface-to-volume ratios, which favor adsorptive phenomena. As crystalline particles become smaller, their surfaces become strained; consequently they have more facets and surface atom steps. This results in atoms in under-coordinated states (i.e., "hot spots") on the surface of NPs to a greater extent than those found in mesoscale materials\textsuperscript{33}. These edge effects lead to differences in chemical reactivity and electronic properties. Although these differences at the nanoscale provide the basis for much opportunity for the development of new industrial and therapeutic products, it is conceivable that materials considered generally bioinert could behave differently at the nanoscale. This behavior may not only be a result of different NP surface properties, but also due to increased ability to be internalized by cell systems through receptor-mediated endocytosis\textsuperscript{34}. Given the commercial promise of nanotechnology, it is necessary to establish whether exposure to manufactured nanoparticles poses significant health risks.

Nanoscale metal oxides such as TiO\textsubscript{2}, ZnO, Fe\textsubscript{3}O\textsubscript{4}, SiO\textsubscript{2}, Al\textsubscript{2}O\textsubscript{3}, and CrO\textsubscript{3} all have been found to have some effect on cellular viability, plasma membrane permeability, cell morphology, lactate dehydrogenase (LDH) membrane leakage, mitochondrial function, and/or increased cell apoptosis\textsuperscript{35; 36}. Some have also been shown to increase inflammatory markers of the endothelium, such as VCAM-1, ICAM-1,
and ELAM-1 and initiate the secretion of proinflammatory cytokines. In previous tests, Al₂O₃ and TiO₂ have shown relatively low cytotoxicity as compared to ZnO and CrO₃, although decreased mitochondrial function and increased LDH leakage occurred at higher NP concentrations. Our laboratory’s recent work with TiO₂, however, has further highlighted the ability of these NPs to induce oxidative stress and inflammation through increased DNA binding of NFκB and phosphorylation of multiple intracellular signaling pathways; physicochemical similarities between TiO₂ and Al₂O₃ indicate the potential for similar health concerns.

Although there are potential negative health concerns associated with nanomaterials, much has been done to limit these potential concerns while building upon the many uses for industrial and biomedical applications. For instance, magnetic nanoparticles (MNPs) have found widespread applications in bioseparation, catalysis, environmental remediation, and magnetic resonance imaging, as well as in emerging medical areas for targeted drug delivery and hyperthermia. Their nanoscale size is critical for their superparamagnetism, and thus their end applications, and much progress has been made in colloidal synthesis for tailoring nanoparticle size and surface characteristics for a wide range of applications. Nanoparticle surface functionalization, and specifically magnetic nanoparticle surface functionalization, with polymer systems is of great emerging interest for combining the beneficial characteristics found at the nanoscale with biomimetic functionalities imparted through polymer science.

1.2 Nutrition as a modulator of environmental chemical insults

1.2.1 Sub-optimal or unhealthy nutrition modulates the toxicity of environmental pollutants

There is a large collection of evidence pointing to the role that a person’s dietary makeup and eating habits can play in the promotion of chronic inflammation, metabolic disorders and vascular diseases. Additionally, emerging data show that exposure to POPs such as PCBs may work in concert with unhealthy diets to promote cumulative or synergistic negative effects. POPs and subprime nutritional status share mechanisms of disease development including the induction of pro-inflammatory pathways. Interestingly, a growing body of evidence implicates an exacerbated toxic effect of PCBs and other environmental pollutants when combined with an additive pro-inflammatory dietary environment. For example, our group has shown that, when combined, pro-
inflammatory PCBs and omega-6 fatty acids create an exacerbated toxicological response that is not simply the sum of their individual inflammatory responses. Importantly, animal fats such as those found in beef and chicken are significant sources of pro-inflammatory medium and long-chain omega-6 fatty acids as well as PCBs and other related POPs. Also, other groups have investigated a linkage between high saturated fat diets and increased toxicity of POPs such as polycyclic aromatic hydrocarbons and have shown an increased risk for adenomas and cancer. In addition, other environmental contaminants such as heavy metals and nanoparticles may work in concert with sub-optimal nutrition to promote chronic inflammation and disease, but studies that investigate the impacts of mixing multiple types of exposures, (e.g. chlorinated pollutants and heavy metals) in combination with the added variable of an unhealthy diet are severely lacking. These types of comprehensive integrated toxicity studies will better mirror real world exposure conditions especially for people residing in close proximity to Superfund and other hazardous waste sites. Newly coined “obesogen research,” which deals with the study of toxicants that can promote obesity and related syndromes, is a compound-driven area of study that is an important addition to the toxicological sciences, but we suggest that it does not focus enough on the interactions between diet and pollutant. Although obesogen research is becoming a more developed area of study, work is still needed to elucidate interactions between nutrients, nutritional status, environmental toxicants and human health.

1.2.2 Healthful nutrition decreases the toxicity of pro-inflammatory pollutants

Polychlorinated biphenyls and related Superfund POPs induce chronic oxidative stress and disregulated inflammatory responses, but anti-inflammatory nutritional antioxidants may buffer and protect against toxicant-induced disease through multiple cell signaling mechanisms. Diets rich in bioactive food components such as polyphenols and omega-3 polyunsaturated fatty acids have been correlated with decreased toxicant-induced maladies including liver diseases, tumor formation and growth, and endothelial cell activation. Polyphenols, for example, are an abundant and diverse class of bioactive compounds found in many fruits and vegetables and have been linked to decreased toxicity from dioxin and dioxin-like PCBs. These reactive oxygen species (ROS) scavenging compounds can also increase PCB excretion rates, prevent AhR-induced inflammation, limit body wasting and decrease cellular dysfunction. Resveratrol, a well-studied polyphenol, has been shown to interact with
the primary receptor of coplanar PCBs, AhR, and to limit its activation and subsequent proinflammatory signaling cascade\textsuperscript{63, 64}. Diets rich in antioxidant and anti-inflammatory agents are well known as a part of French and Mediterranean diets\textsuperscript{65}. It should not be a surprise then that heart disease and related pathologies are significantly lower in these areas when compared to similarly industrialized western regions\textsuperscript{66}. Although it is extremely difficult to determine a unique causative protective agent, bioactive nutrients such as resveratrol, quercetin, tea catechins, and docosahexaenoic acid (DHA) may all work independently or synergistically to decrease oxidative stress, inflammation, and vascular diseases\textsuperscript{67-69}. Since the hallmark of coplanar PCB vascular toxicity is also increased oxidative stress and inflammation, polyphenols (e.g., flavonoids) and omega-3 polyunsaturated fatty acids are prime nutritional candidates as biomodulators of PCB-mediated cytotoxicity. Our work has shown that plant-derived flavonoids such as epigallocatechin-3-gallate (EGCG), and long-chain omega-3 fatty acids such as docosahexaenoic acid (DHA) can protect cellular systems by decreasing pro-inflammatory lipid raft signaling domains called caveolae and by simultaneously up-regulating antioxidant defenses through increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation\textsuperscript{70, 71}. Most recently we have elucidated novel cross-talk mechanisms between caveolae and Nrf2 pathways and have shown that nutrients and/or bioactive food compounds may protect against vascular dysfunction, oxidative stress and inflammation by down-regualting caveolae and simultaneously up-regulating Nrf2 target antioxidant genes\textsuperscript{3}. Other groups have shown that bioactive food compounds such as those found in broccoli (sulforaphane) and red wine (resveratrol) can activate Nrf2 through multiple mechanisms and decrease oxidative stress levels in cells and in animals\textsuperscript{64, 65, 72-75}. Polyunsaturated fatty acids, and especially omega-3 fatty acids, as well as their anti-inflammatory breakdown metabolites can protect against PCB-induced disease by activating Nrf2 but interestingly also by disrupting functional caveolae\textsuperscript{70}. Although caveolae and Nrf2 pathways appear to be prime candidates for bioactive food components to work through, more research is necessary to better elucidate other novel pathways that can be targeted both \textit{in vitro} and \textit{in vivo}.

1.2.3 Protective cellular signaling pathways

Multiple signaling pathways have been attributed to nutritional modulation of environmental toxicants, but we have shown that caveolae and the antioxidant master controller Nrf2 play integral protective roles. There is increasing evidence that lipid raft
membrane microdomains, i.e., caveolae, may play an important role in atherosclerosis, the toxicity of coplanar PCBs, and nutritional protection\textsuperscript{76-78}. Caveolae are flask shaped membrane invaginations that are important in cholesterol transport, nutrient and xenobiotic import into cells and cellular signaling\textsuperscript{79}. Due to the lipophilicity of PCBs, they may enter the cell through lipid raft mediated events or come into contact with caveolae-related signaling proteins. Interestingly, caveolae-mediated endocytosis is a leading mechanism for nanoparticle entry into cells as well and a focal point of nanotoxicology studies (see Figure 1.2)\textsuperscript{80}. Caveolae are abundant in vascular endothelial cells and the cardiovascular system in general, which points to a highly probable role for caveolae in inflammation and atherosclerosis\textsuperscript{81}. Our laboratory has previously reported that coplanar PCBs promote the up-regulation of genes related to the activation of endothelial cells and the initial stages of atherosclerosis, and that the loss of functional caveolae ameliorates these detrimental effects\textsuperscript{82}. Caveolin-1 (Cav-1), the major structural protein of caveolae, contains the “Cav-1 binding domain” that is known to bind multiple proteins including endothelial nitric oxide-synthase (eNOS), v-src sarcoma (SRC), protein kinase C (PKC), extracellular-signal-regulated kinase (ERK) and protein kinase B (Akt); many of which are involved in inflammatory pathways\textsuperscript{76}. For example, down-regulation of Cav-1 can lead to eNOS activation and subsequent increases in diffusible nitric oxide, which has been shown to play a major role in healthy blood pressure and vessel tone\textsuperscript{22}. Many publications from our laboratory and others show down-regulation and elimination of Cav-1 to be anti-atherosclerotic\textsuperscript{23;83}. Importantly, eliminating Cav-1 prevents PCB-induced cellular dysfunction\textsuperscript{82}. Our data point to Cav-1 as a possible anti-atherosclerotic therapeutic target, and we hypothesize that nutritional intervention can down-regulate Cav-1 and, in turn, protect against PCB-induced inflammation.

Nrf2 is a transcription factor that can up-regulate cytoprotective genes in response to oxidative stress, xenobiotics, and bioactive food molecules\textsuperscript{84-86}. Many nutrients, including resveratrol, sulforaphane, and epigallocatechin gallate (EGCG), have been shown to activate Nrf2\textsuperscript{74;87;88}. There are multiple mechanisms of Nrf2 activation, including direct phosphorylation of Nrf2 by PKC delta and loss of contact between Nrf2 and inhibitory kelch-like ECH-associated protein 1 (Keap-1)\textsuperscript{89}. Upon activation, Nrf2 is able to evade ubiquitination, enter the nucleus, and bind cis-acting antioxidant response elements (ARE) in target genes such as heme-oxygenase 1 (HO-1) and NAD(P)H:quinoneoxidoreductase 1 (NQO1)\textsuperscript{90}. Importantly, Nrf2 activation leads to decreased inflammation, which is a hallmark of PCB toxicity\textsuperscript{86}. Activation of Nrf2 may
lead to vascular protection from PCB-induced toxicity, and we hypothesize that a diet rich in bioactive food components can activate Nrf2 and prevent PCB-induced inflammation. Nrf2 has been shown to cross-talk with multiple signaling partners, especially with the major player in PCB toxicity, AhR$^{91}$. Although it has been known for decades that dioxin and dioxin-like compounds activate both AhR- and Nrf2-related genes, it was shown only recently that Nrf2 is required for induction of AhR genes such as CYP1A1$^{92}$. This observed cross-talk can be explained mechanistically at the genetic level by the fact that the promoter region for Nrf2 contains AhR binding regions and the gene promoter for AhR contains multiple Nrf2 binding elements$^{91}$. Our laboratory previously determined that AhR is a binding partner of Cav-1, so it is plausible that cross-talk between caveolae and Nrf2 signaling pathways also exists$^{83}$.

Little is known about the cross-talk between Nrf2 and caveolae signaling and how bioactive compounds such as omega-3 lipids, flavonoids and other polyphenols interact to protect against environmental insults. We have shown that decreasing cellular Cav-1 levels results in a more intense antioxidant response. Mechanistically, we attribute this to decreased AhR activity as well as increased Nrf2 activity$^{60}$. Recently, an intimate example of cross-talk between Cav-1 and Nrf2 was illustrated$^{93}$, and our published and unpublished data supports this phenomenon. We have shown that decreasing Cav-1 via siRNA technology or utilizing Cav-1 KO mice can result in decreased expression of inhibitory Keap1, which allows for increased Nrf2 activation. Importantly, we have shown in vitro that pretreatment with the polyphenol EGCG can cause decreased expression of Cav-1 and up-regulation of the Nrf2 target cytoprotective genes glutathione S-transferase (GST) and NQO1 (unpublished data). We believe that an efficient nutritional biomodulator will work through the observed cross-talk between caveolae and Nrf2 and result in broad and diverse cellular protection. These data support the paradigm that utilizing bioactive food components can be an effective strategy to combat PCB-induced vascular toxicity. Using bioactive food components such as flavonoids and omega-3 polyunsaturated fatty acids (PUFAs) may be an economically and logistically beneficial method to counteract PCB-induced vascular inflammation. Although it does appear that both caveolae and Nrf2 signaling pathways play an important role in nutritional modulation of vascular inflammation, other signaling cascades may also prove to be involved.
Phytochemicals may work through mechanisms that up-regulate protective genes, down-regulate pro-inflammatory genes and/or decrease overall oxidative stress (Figure 1.3). For example, the green tea polyphenol EGCG has been shown to protect against inflammation via many of the previously discussed signaling mechanisms, but interesting new data also points to a role of the laminin receptor. The laminin receptor is a cell surface receptor that has been shown to play a role in inflammation and cancer metastasis, and multiple groups have shown EGCG to directly bind the receptor and subsequently decrease basophil activation, induce apoptosis in cancer cells, inhibit TLR4 and down-regulate pro-inflammatory cytokines and chemokines\textsuperscript{94, 95}. Studying the interactions between laminin receptor, nutrients and environmental toxicants may help to elucidate more effective nutritional modulation strategies to prevent vascular inflammation and disease.

1.2.4 Healthful nutrition can decrease body burden of environmental toxicants

An emerging paradigm implicates healthful nutrition as an effective protective modulator of environmental toxicant-induced inflammation and human disease. Multiple laboratories have investigated the decreased toxicities of environmental pollutants due to bioactive nutrients such as flavonoids and omega-3 polyunsaturated fatty acids, but many of these studies rely on \textit{in vitro} assays that lack the complexity of a whole body organismal approach. Importantly, emerging classes of bioactive food components such as polyphenols also have been shown to modulate the pro-inflammatory effects of environmental toxicants. Our laboratory and others have shown that a wide array of phenolic compounds such as EGCG, curcumin and quercetin can decrease toxicant-induced oxidative stress and inflammation in multiple cell types, tissues and animal species\textsuperscript{54, 59, 67, 71, 96, 97}. Although human studies are lacking, strong evidence in animal models implicates a protective role for flavonoids and other polyphenols perhaps through the induction of antioxidant enzyme pathways and increased fecal excretion rates\textsuperscript{59, 98}. Along with the protective effects of healthful nutrition against biological insults induced by exposure to POPs (summarized in section 1.2.3)\textsuperscript{3}, healthful nutrition, and specifically antioxidant polyphenols, is also being found to decrease body burden of PCBs and other POPs. In other words, plant-derived polyphenols are being found to not only protect against POP-mediated oxidative stress, inflammation, and toxicity but also to bind to POPs and thus contribute to a decrease in body burden. In fact, if has been proposed that green tea, containing high levels of polyphenols including EGCG, can inhibit the
intestinal absorption of lipids and highly lipophilic organic compounds and accelerate excretion of PCBs\textsuperscript{59, 99, 100}.

Certain foods and polluted air can be major sources of toxicant exposures\textsuperscript{8}. Thus, altering nutritional choices may prove to effectively modulate risks associated with exposure to POPs. Making informed dietary decisions such as substituting lower fat versions of protein sources (e.g., legumes, nuts, or lean meats and dairies) may have multiple health benefits including decreased exposure to detrimental pollutants such as dioxins and PCBs\textsuperscript{101}. For many populations it may be difficult or cost-prohibitive to change major dietary protein sources; thus, increasing dietary intake of other bioactive nutrients may buffer already exposed individuals against the negative ramifications of pollutant exposures. For example, several laboratories have shown that diets high in fiber can alter the absorption and excretion rates of pollutants such as PCBs\textsuperscript{102-104}. Mechanistically, multiple dietary fibers have been shown to effectively bind pollutants such as dioxins, which may help to explain increased fecal excretion rates, decrease in body burden, and observed protection\textsuperscript{102}. Increasing excretion of lipophilic toxicants through other nutritional compounds such as fat substitutes may also effectively decrease risk. For example, much emphasis including human clinical trials has been placed on the interactions between the non-absorbable fat substitute olestra and environmental toxicants\textsuperscript{105-109}. Researchers determined that low levels of olestra supplementation (25 grams/day) increased the excretion of multiple PCBs and related contaminants upwards of 11-fold compared to normal diet\textsuperscript{109}. Interestingly, in a human case study, an olestra-supplemented diet completely eliminated excessive POP concentrations in adipose tissue and reversed POP-mediated clinical conditions such as diabetes and hyperlipidemia\textsuperscript{110}. Diet-derived bioactive compounds such as EGCG are attractive modulators of toxic exposure because they may both help to prime the physiological system prior to a toxic insult by up-regulating protective detoxifying enzymes as well as protect therapeutically after exposure by increasing the rate of excretion and lowering overall body burden\textsuperscript{98}. Altering diets to increase fiber and bioactive nutrients from fruits and vegetables is an effective and cost-efficient means of modulating POP toxicity, but interestingly an emerging and growing body of knowledge also implicates the use of bioactive nutrients such as quercetin and curcumin as effective components of novel environmentally friendly remediation technologies.
Environmental remediation not only relies on efficient pollutant degradation but initially on effective pollutant sensing and capture/removal from contaminated settings. Emerging research suggests that the utilization of polyphenols for capture and sensing of POPs may provide a technologically efficient and cost effective remediation platform that results in minimal environmental and health impacts. This work adds to the paradigm that nutrients or diet-derived bioactive compounds can broadly impact health concerns associated with POP exposure (see Figure 1.1).

1.3 Utilizing polyphenols for pollutant sensing, binding, and remediation efforts

1.3.1 Current methods and difficulties for POP sensing and remediation

Persistent organics, and especially PCBs, have proven difficult to remove from contaminated sources and subsequently remediate due to their poor aqueous solubility and low volatility, which makes extraction from soil and water especially challenging\textsuperscript{111}. Further, sensing of these pollutants is both difficult and cost prohibitive, relying heavily on GC-MS methods and complicated extraction techniques\textsuperscript{112-114}. POP remediation to date often involves dredging and subsequent deposition in landfills or further pollutant degradation through incineration or various chemical dehalogenation techniques\textsuperscript{115}. Studies have shown, though, that dredging and deposition results in substantial leaching into the surrounding environment, incineration can produce even more harmful byproducts if insufficient temperatures are reached, and organic solvents used for chemical dehalogenation are often as toxic as the pollutants being remediated\textsuperscript{116, 117}. There is a clear need for more effective pollutant remediation involving inexpensive, environmentally friendly, stable systems for the capture/sensing of PCBs and other POPs, as well as environmentally-conscious pollutant degradation. In fact, as of 2011, the Ecologic Institute of the European Union’s Screening methods for the Water Framework Directive (SWIFT-WFD) has continued to focus on this need for discovery and translation of low-cost screening tools for environmental contaminants\textsuperscript{118}.

As a result, much research has been performed to determine novel technologies and methods to capture, sense, and remediate POPs. One such example includes engineered antibodies that contain binding domains that can specifically associate with PCBs and other POPs, which can be utilized for specific binding and sensing applications\textsuperscript{119}. Due to inherent cost barriers and stability issues of these antibodies, though, other low-cost, stable engineered materials have been sought as an alternative
platform to mimic this specific binding interaction; growing data are implicating the use of nutrient polyphenols as effective components of engineered POP capture/sensing platforms both due to their fluorescent detection characteristics and affinity for chlorinated organics$^{120}$. It has been reported that the binding domains of PCB-specific antibodies (e.g., the monoclonal antibody S2B1) form sterically constrained, highly aromatic pockets, which allow for pi-pi bond stacking interactions$^{119}$. Researchers have successfully mimicked these interactions in the development of synthetic materials, including those composed of polyphenolic moieties, for capture and sensing applications$^{121-124}$. For instance, by exploiting their biomimetic binding properties, researchers have developed porphyrin-containing compounds (highly aromatic compounds with similar molecular geometries to polymeric polyphenols) for the detection of environmental contaminants$^{113; 125}$. 

1.3.2 **Emerging methods for POP sensing and remediation utilizing polyphenol-based nanomaterials**

There is emerging evidence that biomimetic PCB binding domains can be synthesized by incorporating phenolic and related moieties into polymeric coatings, which will greatly enhance the ability to detect and remove/remediate PCB contamination$^{120}$. Polyphenols such as quercetin and curcumin are of special interest not only because of their beneficial health effects and environmentally benign nature but also because of their highly aromatic structures, which may enable effective and/or specific binding and sensing of aromatic pollutants$^{126}$. Recently, we developed a novel strategy to synthesize polymeric networks that incorporate these polyphenols as both stable coating materials and biodegradable hydrogels for use as a pollutant binding platform and an antioxidant release platform to combat localized cellular oxidative stress, respectively$^{127-129}$. These materials can have enhanced biocompatibility over their free compound form$^{130}$ and in preliminary studies, we have shown that PCB binding is increased through the incorporation of phenolic moieties in a polymeric matrix$^4$. Additionally, the characteristic fluorescence peaks associated with these flavonoids effectively have been used preliminarily to sense PCB congeners in a concentration-dependent fashion$^4$.

Although, biomimetic polymeric networks have been utilized for rapid binding of relevant pollutants$^{131}$, these pollutant-bound materials must be easily removed from
contaminated environments in order to be effective remediation systems. Combining polyphenolic polymers with magnetic nanoparticles in nanocomposite microparticle systems, then, allows for rapid binding of aromatic pollutants followed by high-throughput magnetic separation from contaminated samples. Magnetic nanoparticles (MNPs) have found widespread applications in magnetic fluids, catalysis, and bioseparation, and are being utilized increasingly in environmental remediation, either as uncoated particles or polymer-functionalized particles for increased pollutant adsorption, sensing and degradation\textsuperscript{131-134}. Although nanoparticles in general have been studied for some time to elucidate their potential toxicity, magnetic iron oxide nanoparticles have shown minimal cytotoxicity and additionally have been used successfully in biomedical applications for many years in areas such as magnetic resonance imaging, targeted drug delivery, and hyperthermia\textsuperscript{40-43, 135}. Further, recent studies in our group have shown that polyphenolic polymers can enhance the biocompatibility of metal nanoparticles like iron oxide\textsuperscript{136}, thus forming the basis for a safe, environmentally-sound remediation platform. MNPs have found great utility in biomedical applications due to their superparamagnetic properties (conferred by their nanoscale size), which allow for easy manipulation with a magnetic field (e.g., for effective magnetic separation of captured molecules or for transport and immobilization at target sites) and heat generation in response to an alternating magnetic field (AMF) for local heating to modify properties (e.g., reaction rates, binding properties, thermal treatment)\textsuperscript{137}. By utilizing diet-derived polymeric polyphenol coatings of defined thickness and matrix structure, our work has shown that pollutants can be very quickly removed from contaminated water samples\textsuperscript{4}. Further, heating of the material can result in thermal destabilization of the polymer matrix and subsequent release of bound pollutants for potential reuse. This combined system, incorporating the binding potential of nutrient polyphenols with the separation and remote heating potential of magnetic nanoparticles potentially could be used for rapid removal of pollutants from environmental samples. The addition and implementation of bioactive nutrients into engineered polymers and nanoparticles allows for efficient remediation of contaminated sites and is worthy of increased investigation due to the high efficiency and specificity potential as well as the environmentally responsible makeup of the technology.
1.4 Overall research goals

Nutrition is being further substantiated as an important modulator of inflammatory and antioxidant pathways, especially associated with environmental insult, and is also emerging as a tool to address exposure toxicity of persistent organic pollutants as both a sensing and remediation platform (Figure 1.1). Evidence now shows that a person’s nutritional status can play a key role in determining the severity of environmental toxicant-induced pathologies such as diabetes and cardiovascular diseases. People with subprime nutrition may be more susceptible to the toxicity of environmentally persistent pollutants whereas individuals that make healthy nutritional decisions and emphasize the importance of foods high in bioactive nutrients such as flavonoids may be less vulnerable to environmental insults by being able to buffer against the toxicity of pro-inflammatory pollutants. Although augmenting the nutritional profiles of at-risk populations may prove to be an effective modulator of toxicant-induced disease in the long term, it is critical to discover and design novel sensing and remediation technologies that are effective, sustainable and environmentally friendly to address immediate needs. Therefore, the novel and emerging evidence that bioactive nutrients may be incorporated into sensing and remediation components may lead to viable technologies that circumvent many of the issues concerning current-day decontamination platforms. Ultimately, further research into nutrient polyphenols, such as EGCG, quercetin, and curcumin, will highlight their potential as sustainable, inexpensive components both that can decrease health concerns associated with environmental pollutants in the short term and remediate these concerns in the long term.

The following studies highlight a systematic approach to addressing concerns associated with POP toxicity through both environmental remediation efforts and biomedical intervention strategies. PCBs 77 and 126 were selected as examples of coplanar persistent environmental pollutants that exhibit dioxin-like toxicity. PCB degradation product in vitro toxicity was evaluated following pollutant dechlorination using iron/palladium nanoparticles, as was in vitro toxicity attributed to other metal oxide nanoparticles, specifically titanium dioxide nanoparticles. Improved methods for nanoparticle suspension for subsequent industrial application and toxicity evaluation were also studied. Biomedical intervention strategies were developed using nutrient polyphenols to mitigate in vivo PCB toxicity and polyphenol-functionalized nanoparticles were developed as a biologically-inspired remediation platform for removing PCBs from
contaminated water sources. Overall, this research has sought to address the significant public health risks and enduring remediation challenges posed by PCBs as model toxicants, as well as to further understand potential concerns and opportunities associated with nanomaterial usage. (The information in Chapter 1 represents a compilation of information from published review articles.)
**Figure 1.1.** Illustration of nutrition as 1) a modulator of health risks associated with environmental exposure and toxicity of persistent organic pollutants (POPs) and as 2) an environmentally sustainable tool to capture, sense and remediate POPs. We propose that nutrition, and in particular diet-derived bioactive compounds such as polyphenols, can protect against POP toxicity through their antioxidant and anti-inflammatory properties. Polyphenols also can participate in a remediation platform that includes their roles in POP capture and sensing technologies, thus allowing for environmentally friendly and sustainable remediation of POPs.
Figure 1.2. Three mechanisms of endocytosis: clathrin-mediated endocytosis (CME), caveolae-mediated uptake (CAV), and clathrin- and caveolae-independent internalization (CCIE).
Figure 1.3. Caveolae play a critical role in PCB-induced vascular toxicity. Protective bioactive nutrients such as omega-3 PUFAs and flavonoids can utilize caveolae Nrf2 cross-talk to decrease reactive oxygen species, limit inflammatory responses and ultimately prevent PCB-induced vascular toxicity.
Chapter 2. Optimization of alumina nanoparticle dispersion for environmental and toxicological studies

2.1 Synopsis

Current dispersion techniques of nanoparticles (NPs) rely on organic solvents, pH extremes, or stabilizing agents, but these methods are not conducive to environmental and toxicological studies. The aim of this study was to determine optimal dispersion characteristics for nano-sized aluminum oxide (alumina) particles in aqueous solutions that mimic biological systems. Alumina NP dispersion was tested in six biologically relevant buffers, as well as cell culture media, and artificial pulmonary surfactant. Suspension media, concentration, and pH were optimized for preparation of alumina NPs at or below 100 nm in size. These NPs were suspended most stably and reproducibly in citrate buffer using high-shear probe sonication (forming particle sizes of ~68 nm), and exhibited high charge stabilization, which produced highly stable suspensions. Acetate buffer also served to suspend alumina at approximately 95 nm in size, although higher polydispersity and decreased long-term suspension stability hampered its use. Alumina particles initially prepared using citrate buffer also remained well suspended with size near 70 nm following addition to cell culture media, indicating that this may be a valuable nanoparticle preparation mechanism for further toxicological studies examining nano-sized effects, instead of the more typical studies associated with micron-sized particle agglomerates. This research highlights mechanisms to stably and reliably disperse alumina NPs in the nano-size regime for environmental and toxicological studies.

2.2 Introduction

Nanotechnology has become commonplace not only in the scientific community, but also in industry and the consumer market. Nanoscale aluminum oxide (alumina) has become important for its wide range of uses: water- and wear-resistant coatings, propellants, personal care products, pharmaceuticals, air and water purification, and structural ceramics, to name a few. There are approximately 50 million tons of alumina produced each year, which are increasingly being used in nanoparticle (NP) form. This increased use of nanoscale alumina has in turn led to increased concern over its safety. Although much is known about alumina on a macroscopic level, little is
known about the toxicological properties of its NP form. Most metal oxides, including alumina, are benign at the macroscale, and even at the microscale, but, because of quantum size effects and larger surface area, as the nanoscale is approached materials can exhibit drastically different properties\textsuperscript{143; 144}.

Nanoparticles involve a size range between 1 and 100 nm. Nanopowders, on the other hand, consist of agglomerates of these NPs that can begin to exhibit properties of the bulk material. Although the manufactured size of NPs is often 10-50 nm, the agglomerates formed from these particles can easily reach to micron sizes, limiting the material's benefits found at the nanoscale\textsuperscript{145-148}. Effective stabilization of alumina NPs, especially in aqueous environments, allows for their further utilization in biomedical applications but has proven difficult because most industrial NPs have not been developed to be compatible with physiological or environmental conditions\textsuperscript{149}. Instead, they rely on organic solvents, pH extremes, or stabilizing agents to remain in suspension\textsuperscript{147; 150-160}. In order to maintain efficient particle dispersion, the steric or charge barriers that cause particle-particle repulsions must be sufficient to overcome the attractive van der Waals forces that lead to particle adherence\textsuperscript{161; 162}. As particles agglomerate, these larger particles gain an increased affinity for smaller particles, eventually resulting in bulk material formation that sediments out of suspension\textsuperscript{163; 164}. Dispersant layers (adlayers) surrounding the NPs of interest are often used to provide a steric barrier when using NPs in biologically relevant environments and are effective at maintaining stable NP suspensions\textsuperscript{159; 165}. In order to study the actual environmental and physiological effects of NPs instead of that simply attributed to their adlayers, it is necessary to obtain effectively stabilized NP suspensions both under near-neutral pH conditions in biologically relevant buffers and without surface passivation by materials not found in nature or in the body\textsuperscript{166; 167}.

Analysis of NPs in suspension provides another obstacle. Transmission electron microscopy (TEM) is often used to provide highly accurate particle size determination, but cannot be used to determine overall aggregation characteristics or gather information about how particles behave in solution. Dynamic light scattering (DLS), on the other hand, can be used to determine average hydrodynamic diameters of NPs interacting in solution\textsuperscript{168; 169}. Laser Doppler electrophoresis (LDE) also can provide an indication of the stability of a suspension by measuring its zeta potential and electrophoretic mobility\textsuperscript{169}. At high zeta potentials, either positive or negative, charge
stabilization occurs between the bulk solution and the nearby particles causing the suspension to resist aggregation. By tailoring the suspension medium with regard to concentration and the types of ions in solution, it is possible to obtain a stable suspension of NPs that could resist aggregation.\textsuperscript{170}

Although there is much potential for NPs to drastically improve biomedical and consumer products, they also present potential physiological and toxicological concerns because, for one, they are capable of passing through cell membranes and into the cytosol of cells.\textsuperscript{2} Principally, their very small size, but also their shape, chemical composition, and surface charge, facilitates entry into the body and into cells. It is thought that NPs enter cells through active transport mechanisms such as receptor-mediated endocytosis, but are also capable of passing into cells passively via pinocytosis.\textsuperscript{171-175} Nanoscale metal oxides such as TiO\textsubscript{2}, ZnO, Fe\textsubscript{3}O\textsubscript{4}, SiO\textsubscript{2}, Al\textsubscript{2}O\textsubscript{3}, and CrO\textsubscript{3} all have been found to have some effect on cellular viability, plasma membrane permeability, cell morphology, lactate dehydrogenase (LDH) membrane leakage, mitochondrial function, and/or increased cell apoptosis.\textsuperscript{35,36} Some have also been shown to increase inflammatory markers of the endothelium, such as VCAM-1, ICAM-1, and ELAM-1 and initiate the secretion of proinflammatory cytokines.\textsuperscript{37} In previous tests, Al\textsubscript{2}O\textsubscript{3} and TiO\textsubscript{2} have shown relatively low cytotoxicity as compared to ZnO and CrO\textsubscript{3}, although decreased mitochondrial function and increased LDH leakage occurred at higher NP concentrations.\textsuperscript{36,38} Our laboratory’s recent work with TiO\textsubscript{2}, however, has further highlighted the ability of these NPs to induce oxidative stress and inflammation through increased DNA binding of NFκB and phosphorylation of multiple intracellular signaling pathways; physicochemical similarities between TiO\textsubscript{2} and Al\textsubscript{2}O\textsubscript{3} indicate the potential for similar health concerns.\textsuperscript{39}

Recent tests performed with alumina use NPs with manufactured diameters of approximately 20-50 nm. However, it is broadly known that in aqueous solutions manufactured NPs exist as aggregates with size of 200-500 nm and above.\textsuperscript{37,140} Sonication is commonly used to disperse nanopowders as smaller and smaller aggregates, but proper charge stabilization following dispersion is key for maintaining effectively dispersed NP suspensions.\textsuperscript{176} Stabilization of NPs nearer to their manufactured size could result in greater effectiveness in pharmaceutical/consumer applications, but also potentially increased toxicity and additional ability of inhaled nanoparticulates to progress further down the respiratory tract.\textsuperscript{177-179} Tests with soot
samples have shown that particles ranging from approximately 100 nm to 20 µm can deposit in nasopharyngeal/laryngeal tissue, while particles less than 100 nm are able to reach alveolar tissue. At the alveoli, NPs are not only able to interact with type I and II alveolar cells, but there is some concern that they may be able to translocate from the respiratory system via the alveoli to the capillaries and bloodstream. It is therefore important to investigate the physiological impact of particles stabilized at or below 100 nm. These tests in biologically relevant systems are meant to shed light on improved methods of alumina NP dispersion not only for potential use in biomedical and consumer applications, but also for continued risk assessment regarding cellular interactions with NPs, and the nanotoxicological effects thereof.

2.3 Methods and Materials

2.3.1 Materials

Commercial grade uncoated Al₂O₃ NPs were obtained from Alfa Aesar (0.01-0.02 µm, Lot # B24Q09, Ward Hill, MA) and initially characterized, as seen in Table 2.1. Sodium acetate, acetic acid, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate, tris(hydroxymethyl)aminomethane carbonate (Trizma base), 2-(N-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), citric acid, sodium citrate, dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were reagent grade or better, and all aqueous solutions were prepared using 16 MΩ deionized water from a Milli-Q water purification system (Millipore, Bedford, MA). Dulbecco’s Modified Eagle Medium (DMEM)/High Glucose and fetal bovine serum (FBS) were purchased from ThermoFisher Scientific (Waltham, MA) and F-12K, 1X media were obtained from Mediatech (Manassas, VA). Copper Lacey Carbon TEM grids (200 mesh) were obtained from Electron Microscopy Services (Hatfield, PA).

2.3.2 Experimental conditions and measurement techniques

Alumina (Al₂O₃) nanoparticle size, surface area, and purity were initially evaluated in comparison to vendor specifications. Elemental analysis of Al₂O₃ samples (prepared at 100 ppm in deionized H₂O and evaporated to dryness on carbon tape) was performed using a Hitachi S-4300 scanning electron microscope (Houston, TX) with a Princeton Gamma-Tech energy dispersive X-ray spectroscopy (EDS) Microanalysis.
System (Princeton, NJ), primary and aggregate particle sizes were determined using scanning electron microscopy (SEM), and surface area was determined through the Brunauer-Emmett-Teller (BET) method using a Micromeritics TriStar 3000 surface area and pore size analyzer (Norcross, GA), following overnight degassing at 120 °C. Initial Al₂O₃ nanoparticle dispersion and size characteristics (suspended in deionized water at 100 ppm) were analyzed using a Malvern ZetaSizer Nano DLS/Zeta potential system with DTS Nano 5.1 Dispersion Technology software (Worcestershire, United Kingdom). Characterization results can be found in Table 2.1 as compared to vendor supplied values.

Herein, alumina nanoparticles were initially suspended at 100 ppm in suspension medium of interest (n≥6). Samples were vortexed to initially suspend particles prior to ultrasonating in an ultrasonic bath (model #: B3500A-MTH, rated output: 135W, 42 KHz, VWR, Radnor, PA) or with a sonic dismembrator probe sonicator (model #: XL2015, rated output: 475W, 20KHz, 1” probe immersion depth, pulsed at 15% duty, power input: 50W, Qsonica, Newtown, CT). Samples were sonicated in ice/water baths to maintain temperature and limit adverse affects from sample heating. Nanoparticle suspensions were filtered through 0.2 µm Anodisc 47 membrane filters (Whatman, Maidstone, United Kingdom) or centrifuged using a Beckman Coulter Avanti J-251 Ultracentrifuge (Fullerton, CA), depending on suspension mechanism, to separate any remaining large aggregates and sedimented particles. Sample NP final concentrations were confirmed using a Thermo iCAP 6500 Duo inductively coupled plasma optical emission spectrometer (ICP-OES) with iTeva ICP software (Waltham, MA). Sample size and NP physicochemical characteristics were analyzed using a Jeol 2010F transmission electron microscope with an Oxford EDS detector (Tokyo, Japan). Nanoparticle hydrodynamic diameters and suspension zeta potentials were analyzed via DLS analysis.

### 2.3.3 Suspension of nanoparticles in buffers

Various buffers and preparation conditions were tested to determine alumina NP dispersion conditions. Alumina was suspended in buffers with pKₐ values covering a wide pH range from pH 4 to 9. Acetate buffer was prepared in deionized water at 0.1, 0.01, and 0.0001 M concentrations, pH 4, using sodium acetate and acetic acid. Acetate buffer was selected due to previous success suspending metal oxide NPs. Citrate buffer was prepared at 0.1, 0.01, and 0.0001 M concentrations, pH 4 and 6, using sodium
citrate and citric acid. Buffer solutions of 0.5 M MES and 1 M HEPES were tested at pH 5.5 and 7.5, respectively. PBS 1X was tested due to its wide use and physiological pH, which is conducive for cell culture studies. One molar Tris buffers (using Trizma base) were prepared at pH 7.5, 8.6, and 9 in order to analyze solution stabilization characteristics at, above, and below the pKₐ of alumina’s surface hydroxyls (pKₐ 8.6 in water)\(^\text{181, 182}\). Alumina samples were bath sonicated for 6-, 12- or 24-h increments or probe sonicated for 10 min. Following ultrasonication, samples were allowed to sit undisturbed for 30 min in order to determine buffer suspension capabilities. In the case of rapid visible sedimentation, the buffer was eliminated from further studies. Subsequently, filtered samples were compared to unprocessed samples to determine the effects of vortexing and filtration on resultant concentration (determined by ICP-OES), overall particle size (determined by DLS and TEM), and suspension stability (inferred visually and by zeta potential). The following suspension conditions were compared among the various buffers: buffer concentration, pH, dispersion method and duration, and suspension stability (see Table 2.2 for alumina suspension conditions).

2.3.4 Suspension of nanoparticles in artificial pulmonary surfactant

Lung surfactant was simulated using 10 mg/mL DPPC in deionized water in order to compare suspension characteristics between buffered systems and a more physiological environment. Although this phospholipid can be solubilized in chloroform or 90% propanol (v/v), these dispersants can act as cell fixatives and are not appropriate further for cell culture studies. A previously reported DPPC dispersion medium was also used as another means of forming stable alumina NP dispersions in the nano-sized regime\(^\text{183, 184}\). This dispersion medium was prepared in deionized water with 0.6 mg/mL BSA and 0.01 mg/mL DPPC and adjusted to pH 7.4. Deionized water was used as an alternative to PBS, which was used in previous studies, to address potential issues with phosphate-induced alumina aggregation. Alumina was suspended with vortexing for 4 h at room temperature followed by bath sonication for 3 min. DPPC contains two 16-carbon chains that can be cleaved by prolonged sonication; limited sonication serves to solubilize the phospholipid more effectively while limiting breakdown of the molecule. Alumina suspensions were centrifuged at 4000 rpm for 30 min at room temperature to remove any remaining large agglomerates.
2.3.5 Suspension of nanoparticles in media

DMEM/High Glucose media and F-12K media were tested as alumina stabilizing agents due to their applicability for a variety of cell types, especially alveolar cells. Alumina nanoparticles were initially prepared as above in biologically relevant buffer and subsequently added to media at approximately 30 ppm final alumina concentration, in line with previously published alumina toxicology study concentrations\(^{35}\).

2.4 Results and Discussion

2.4.1 Determination of optimal particle dispersant

Alumina NPs, with a manufactured size of 20 nm, were suspended in six buffers at an initial concentration of 100 ppm (See Table 2.2). Particle size analysis is contingent on Brownian motion; therefore, extensive sedimentation leads to decreased reliability of sizing results. Optimal buffer dispersant was then chosen based on its ability to both effectively suspend alumina NPs and limit particle aggregation. Optimal preparation conditions were chosen based on resultant NP size. The stability of effectively dispersed samples was monitored for 2 weeks by analyzing the change in particle size and zeta potential. Number distribution was primarily used in our DLS studies in order to obtain a more reliable analysis of multimodal systems made up of a mixture of individual particles and small aggregates as well as larger particle agglomerates. One must take into account, though, that because the number distribution is an estimation – as this system is not an actual particle counter – it is not completely reliable on its own to give information about the system. Consequently, although number distribution was primarily used for analysis, data were also compared to intensity distribution DLS measurements and electron microscopy results.

Four buffers, including 0.1 M citrate (pH 6.0), 0.1 M citrate (pH 4.0), 0.1 M acetate (pH 4.0), and 1 M HEPES (pH 7.5), were capable of suspending alumina to varying extents, while 1X PBS (pH 7.4), 0.5 M MES (pH 6.0), and 1M Tris (pH 7.5, 8.6, and 9) buffers were unable to stabilize alumina enough to obtain reliable size measurements. Initial measurements were performed following 6 h sonication in an ultrasonic bath at 25 °C. Although 0.1 M citrate, pH 4, was capable of dispersing alumina NPs, the presence of high particle polydispersity indicated poor particle stabilization. HEPES buffer stabilized particle aggregates outside the target size range with aggregate size being approximately 1.8 \(\mu\)m. Number distribution was used to look
more specifically at smaller particle peaks. Peak alumina NP sizes (hydrodynamic particle diameter) in citrate and acetate buffers were 90±1 nm and 101±7 nm, respectively. Although acetate buffer, pH 4, produced particle aggregates in the target size range (~70-100 nm), it also exhibited much increased size variability between samples because of poor particle charge stabilization. Use of citrate buffer, pH 6, as an alumina suspension medium provided small agglomerates, highly reproducible results, and very little formation of large aggregates.

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to determine the effect of filtration on sample concentration following initial sample preparation. Alumina dispersed in citrate buffer, pH 6.0, with 10 min probe sonication showed average alumina NP recoveries (filtered/unfiltered %) of 27%, indicating final sample concentrations of approximately 27 ppm alumina NPs dispersed in buffer, which is in line with recent concentrations used for toxicology studies. Contaminants due to the citrate buffer were assumed to be minimal in comparison to alumina NPs for the purpose of sample analyses.

Although not reported, deionized water was also tested as an alumina suspension medium control (see initial particle characterization in Table 2.1). A bimodal particle size distribution was exhibited, with both particles approximately 140 nm in size as well as large, micron-sized aggregates, although large aggregates heavily predominated. PBS does not stabilize alumina because phosphate groups bind rapidly and strongly to the surfaces of particle aggregates, thus limiting particle disaggregation during sonication. It is presumed that Tris buffer (pH 7.5, 8.6, and 9) is unable to stabilize alumina NPs because pH proximity to the pKₐ of alumina produces a very small electric potential difference between the buffer and the particles, resulting in very small zeta potential and diminished charge stabilization.

2.4.2 Buffer concentration effects on particle dispersion

Acetate and citrate buffers (pH 4.0 and 6.0, respectively) were tested at concentrations of 0.1, 0.01, and 0.0001 M after 6 h sonication durations using both number and intensity distribution DLS analysis. It was found that higher concentrations yielded overall lower particle sizes. Nanoparticle suspension in acetate buffer produced higher sample variability as compared to citrate buffer, and, although a small proportion of agglomerated particles were stabilized at approximately 100 nm size, large particle
formation occurred below 0.1 M resulting in a majority of aggregates being approximately 1 µm in size.

With alumina NPs suspended in citrate buffer, though, there is a correlation between concentration and particle size (see Figure 2.1). Higher buffer concentrations led to both a decrease in NP aggregate size (hydrodynamic diameter) and polydispersity; alumina NPs suspended in 0.1 M citrate had the lowest polydispersity and smallest overall particle size of 109±4 nm following 6 h sonication in an ultrasonic bath at 25 °C. Polydispersity increased, with a decrease in suspension stability, at lower buffer concentrations. The citrate anion, with its high overall charge at pH 6, presumably forms a highly stable electric double layer around suspended alumina NPs that limits particle-particle interactions, resulting in a highly stable dispersion.

2.4.3 Ultrasonication effects on particle dispersion

It is well known that sonication conditions help to disperse nanoparticles more effectively, but this process can be reversed if the surface charge of newly fractured nanoagglomerates is not maintained to prevent reaggregation. As such, inefficient suspension media were removed from further optimization studies. A concentration of 0.1 M citrate buffer, pH 6.0, subsequently was tested under various ultrasonication conditions in order to further optimize particle size and dispersion stability. Alumina NPs were ultrasonicated in buffer for 6, 12 and 24 h at 25 °C in an ultrasonic bath, and for 10 min on ice with a sonic dismembrator probe sonicator. A trend toward smaller particles was seen when ultrasonication duration was increased, while probe sonication yielded the smallest peak aggregate diameters (see Figure 2.2). Mean particle sizes ranged from 109±4 nm for 6-h sonication to 68±9 nm for 10-min probe sonication. The intensity distribution showed the presence of aggregates, approximately 1 µm in size, which decreased from 9.8% of the total sample after 6 h of sonication to 0.6% after 24 h. Increased sonication time allows for increased disaggregation; smaller particles disaggregate to a lesser extent because of increased particle-particle adhesive forces found at small overall particle diameters. The sonic dismembrator instead relies on higher power output and proximity of the probe to the sample to produce smaller particle aggregates with drastically reduced ultrasonication durations.

Samples were compared to unprocessed samples to determine the role that ultrasonication plays in forming stable alumina suspensions in the target size regime.
Data confirm findings of Oesterling et al. 2008 that unprocessed samples form aggregates of approximately 200-500 nm or above in aqueous systems, and our studies of unprocessed samples in citrate buffer, pH 6.0, exhibit approximate aggregate sizes of 200-300 nm, which drastically decrease with limited sonication.

### 2.4.4 Particle suspension stability

As seen in Figure 2.3, citrate buffer produces substantial alumina stability at various particle sizes. Alumina NP stability in 0.1 M citrate buffer, pH 6.0, was tested over 2 weeks following sample preparation with 10 min (probe sonicator) and 6 and 24 h (ultrasonic bath) ultrasonication periods. In each case, alumina aggregate size remained essentially constant (at least within 6.1% of the initially measured aggregate size), indicating that 0.1 M citrate buffer is capable of maintaining well-dispersed suspensions of various-sized alumina NPs based on the extent of initial ultrasonication.

Figure 2.3 represents data analyzed as a number distribution in order to more closely analyze data in the target size regime. Although the overall suspension remained stable during the testing period, intensity distribution results showed a small concentration of larger aggregates, approximately 500-1000 nm in size that increased over time from 0.5% of the initial suspension to at most 24% of the suspension after 2 weeks. As with the initial sonication duration testing, samples remained stable with specific NP sizes based on the extent of sonication.

Zeta potential measurements were also used to infer particle stability, based on surface charge determination, in various suspension media (see Table 2.3 for results). Zeta potential represents the charge stabilization between the electric double layers of ions surrounding the suspended particles with the bulk solution, and therefore is indicative of the overall charge stability of the dispersion. As zeta potential increases, in either the positive or negative direction, the charge repulsion between particles and the bulk solution also increases; thus, higher zeta potential values represent more stable overall suspensions. Particles suspended in citrate buffer had the highest zeta potential values, and thus the highest stability based on charge, which corresponds well with NP stability as seen in DLS measurements. All DLS results dealing with particle stability were confirmed with zeta potential analysis, indicating citrate buffer as the most stable suspension medium.
2.4.5 Transmission electron microscopy analysis

Transmission electron microscopy (TEM) analysis of samples showed alumina particle aggregate sizes that correspond to aggregate diameters found using DLS analysis. Alumina dispersed in citrate buffer, pH 6.0, with 10 min probe sonication showed average aggregate size of ~72 nm, while alumina suspended via bath sonication with 24 h sonication and 6 h sonication resulted in aggregate diameters of ~84 nm and 113 nm, respectively. High dilution samples were analyzed to minimize potential drying effects that result in larger aggregate formation. Because TEM confirmed the sizes obtained through DLS, additional aggregation due to drying is likely minimal (See Supplementary Figure 2.1).

2.4.6 Particle suspension in artificial pulmonary surfactant

Alumina NP suspension characteristics were also tested in systems simulating physiological environments to facilitate toxicological studies. In the case of inhalation of alumina NPs into the lungs, alumina will first come into contact with pulmonary surfactant, composed primarily of dipalmitoylphosphatidylcholine (DPPC), as well as phosphatidylglycerol (PG) and small quantities of surfactant proteins. DPPC suspended in deionized water at 10 mg/mL was used to simulate pulmonary surfactant found in the respiratory tract and specifically in the alveoli. Additionally, dispersion medium consisting of a lower concentration of DPPC (0.01 mg/mL) and BSA was used as an artificial system to mimic alveolar pulmonary surfactant while limiting adverse affects of high protein and phospholipid content on cellular viability NP size measurement in DLS. When larger particles, greater than approximately 10 µm in size, enter the respiratory tract they are immediately covered in mucus and removed via the mucocilliary escalator. Smaller particles that are approximately 1-10 µm in size are able to traverse to the lungs where they most likely interact with pulmonary surfactant and are cleared. Nanoparticles, though, have the potential to reach the alveoli and interact with alveolar cells as well as the alveolo-capillary barrier separating the pulmonary and circulatory systems instead of readily being cleared from the respiratory tract\textsuperscript{180, 187-189}. It is thought that if NPs aggregate in artificial lung surfactant, they will also tend to aggregate \textit{in vivo}. These aggregates are more easily cleared from the lungs, thus limiting the adverse affects of nanoparticulates. In the case that alumina remains well suspended in lung surfactant, toxicological concern increases because of the ability of NPs to enter cells.
and cellular organelles. It is possible that lung surfactant can instead serve to coat and stabilize very small NPs.

At concentrations above the critical micelle concentration (CMC, $\sim 3.4 \times 10^{-7}$ mg/mL$^{190}$), DPPC forms micelle particles in water, which leads to the presence of a DPPC particle peak when measured by DLS$^{191,192}$. In our DLS studies, DPPC exhibited a particle diameter of $146 \pm 31$ nm in the absence of alumina NPs. In multiple tests, the presence of 100 ppm alumina NPs in the DPPC/H$_2$O suspension caused a decrease in the observed particle diameter from $146 \pm 31$ nm without alumina to $79 \pm 9$ nm with alumina (see Figure 2.4). It is possible that a sufficient amount of the DPPC adsorbs to the surface of the alumina NPs in the suspension to decrease the DPPC concentration below its CMC and prevent micelle formation, which would account for the absence of a DLS peak representing DPPC in the presence of alumina NPs. In this case, it is probable that the DPPC does indeed stabilize alumina NPs at approximately 80 nm, although this particle size range represents only one-quarter of the overall suspension while larger aggregates (assessed through intensity distribution DLS analysis) predominate. The presence of a high polydispersity index indicates that, although DPPC may have the ability to stabilize alumina as relatively small aggregates, the overall stability of the suspension is low.

In contrast to the effectively dispersed titania seen previously using DPPC/BSA dispersion medium, alumina dispersion stability remained similar to that of DPPC alone in water at higher phospholipid concentrations ($\sim 200$ nm alumina aggregate diameters)$^{183,184,193}$. Although it would have been beneficial to test NP suspension characteristics in artificial pulmonary surfactant with higher energy dispersion techniques (i.e., bath or probe sonication), vortexing was used herein to limit DPPC phospholipid breakdown and subsequent unreliable sample analysis.

Alumina NP stability was tested in DPPC/water for three weeks (data not shown); high particle size variability is present within the first week, which increases drastically after one week of suspension at which point aggregated particles predominate. Although number analysis indicates that some particles range from 60 to 90 nm in size, high polydispersity indices indicate that DPPC/water does not suspend particles reliably or for long-term.
2.4.7 Particle dispersion in cell culture media

Alumina dispersions in DMEM/High Glucose and F-12K media produce similar obstacles to DLS analysis because of interference of various proteins and macromolecules in the target size range of the alumina particles. TEM analysis showed that, while DMEM/High Glucose and F-12K media do not serve as sufficient initial particle dispersants, alumina dispersed initially in 0.1 M citrate buffer, pH 6.0, remains in a target size range following addition to media at 30 ppm. TEM analysis of citrate-stabilized alumina, which was suspended in DMEM/High Glucose medium, showed primary particle sizes of approximately 93 nm, although larger aggregates were also present (~150 nm in size). F-12K medium effectively suspended citrate-stabilized alumina at particle diameters of approximately 79 nm with limited aggregates of ~120 nm in size. Consequently, F-12K and DMEM/High Glucose media are well suited to suspension of citrate-stabilized nanoparticulate alumina for subsequent cell culture toxicology studies.

2.5 Conclusion

This work highlights the optimization of, as well as the many difficulties associated with, alumina nanoparticle suspension in aqueous systems for toxicological evaluation of alumina effectively stabilized in the nano-sized regime (particle sizes at or below 100 nm). Alumina NPs were dispersed in a wide range of physiologically relevant buffers, cell culture media, and artificial pulmonary surfactant model systems. These NPs were suspended most stably and reproducibly in citrate buffer using high-shear probe sonication (forming particle diameters near 70 nm), and exhibited high charge stabilization, which produced highly stable suspensions. Alumina particles initially prepared using citrate buffer also remained well suspended near 70 nm following addition to cell culture media, indicating that this may be a valuable nanoparticle preparation mechanism for further toxicological studies examining nano-sized effects, instead of the more typical studies associated with micron-sized particle agglomerates. Further toxicological studies of alumina stabilized in the nano-sized regime will be of ongoing importance as these particles are increasingly used in industrial, environmental, and personal care settings. (The data presented in Chapter 2 has been submitted for publication)

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<th>Vendor purity</th>
<th>Vendor surface area (m²/g)</th>
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<th>Analyzed DLS Avg. aggregate size (size range) (nm)</th>
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¹Initial alumina NP SEM and DLS analyses were performed at 100 ppm in deionized H₂O. For SEM, H₂O suspension medium was evaporated to dryness prior to analysis.
Increased concentration-related conditions were tested for citrate and acetate buffers due to higher dispersion efficiencies and to test ionic strength effects.

Bath and probe ultrasonication techniques (listed as "bath" and "probe"), as well as vortex mixing, were used to suspend alumina NPs in suspension media.

1X PBS consisted of 137 mM NaCl, 12 mM phosphate and 2.7 mM KCl

<table>
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<tr>
<th>Suspension agent</th>
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<th>pH</th>
<th>Preparation conditions&lt;sup&gt;2&lt;/sup&gt;</th>
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<sup>1</sup>Increased concentration-related conditions were tested for citrate and acetate buffers due to higher dispersion efficiencies and to test ionic strength effects.

<sup>2</sup>Bath and probe ultrasonication techniques (listed as "bath" and "probe"), as well as vortex mixing, were used to suspend alumina NPs in suspension media.

<sup>3</sup>1X PBS consisted of 137 mM NaCl, 12 mM phosphate and 2.7 mM KCl.
<table>
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Figure 2.1. (A) Number and (B) intensity distributions of alumina nanoparticle dispersion in citrate buffer, pH 6.0, prepared using 6 h bath sonication at 25 °C. Increasing buffer concentration yields an overall decrease in particle agglomerate diameter. Intensity distribution and undersize data of alumina NPs suspended in 0.1 M citrate buffer indicate minimal large aggregate formation and homology with number distribution data.
Figure 2.2. Number distribution of nanoparticle dispersion in 0.1 M citrate buffer, pH 6.0, with varying sonication conditions at room temperature. When prepared using an ultrasonic bath, mean particle size decreases with increasing sonication duration from 109±4 nm for 6 h sonication to 78±3 nm for 24 h sonication. Increasing sheer using probe sonication produced the smallest overall alumina aggregate diameters at 68±9 nm.
Figure 2.3. Alumina nanoparticle stability in 0.1 M citrate buffer, pH 6.0, was tested for 2 weeks after sample preparation using bath and probe sonication. Each preparation condition provides relatively long-term mean sample stability, with a change in sample diameter of +5±4 nm (6 h bath sonication), -3±5 nm (24 h bath sonication), and +5±2 nm (10 min probe sonication).
Figure 2.4. Alumina particle stabilization in dipalmitoylphosphatidylcholine (DPPC). Size analysis of alumina nanoparticles suspended in 10 mg/mL DPPC. DPPC exhibits its own peak at approximately 150 nm, in the absence of alumina nanoparticles, corresponding to micelle formation.
Chapter 3.A. Titanium dioxide nanoparticles increase inflammatory responses in vascular endothelial cells

3.A.1 Synopsis

Atherosclerosis is a chronic inflammatory disease that remains the leading cause of death in the United States. Numerous risk factors for endothelial cell inflammation and the development of atherosclerosis have been identified, including inhalation of ultrafine particles. Recently, engineered nanoparticles (NPs) such as titanium (TiO$_2$) NPs have attracted much attention due to their wide range of applications. However, there are also great concerns surrounding potential adverse health effects in vascular systems. Although TiO$_2$ NPs are known to induce oxidative stress and inflammation, the associated signaling pathways have not been well studied. The focus of this work, therefore, deals with examination of the cellular signaling pathways responsible for TiO$_2$ NP-induced endothelial oxidative stress and inflammation. In this study, primary vascular endothelial cells were treated with TiO$_2$ NPs for 2-16 h at concentrations of 0-50 µg/mL. TiO$_2$ NP exposure increased cellular oxidative stress and DNA binding of NFκB. Further, phosphorylation of Akt, ERK, JNK and p38 was increased in cells exposed to TiO$_2$ NPs. TiO$_2$ NPs also significantly increased induction of mRNA and protein levels of vascular cell adhesion molecule-1 (VCAM-1) and mRNA levels of monocyte chemoattractant protein-1 (MCP-1). Pretreatment with inhibitors for NFκB (pyrrolidine dithiocarbamate), oxidative stress (epigallocatechin gallate and apocynin), Akt (LY294002), ERK (PD98059), JNK (SP600125) and p38 (SB203580) significantly attenuated TiO$_2$ NP-induced MCP-1 and VCAM-1 gene expression, as well as activation of NFκB. These data indicate that TiO$_2$ NPs can induce endothelial inflammatory responses via redox-sensitive cellular signaling pathways.

3.A.2 Introduction

Nanotechnology and the production of nano-sized particles have emerged as promising areas of study due to their many applications in industry and medicine$^{29,30}$. Particularly, TiO$_2$ NPs are produced on a large scale and are being employed in a variety of consumer products, such as sunscreens, cosmetics, pharmaceutical additives and food colorants$^{194,195}$. Although TiO$_2$ is considered to be a safe material, concerns have been raised about the potential adverse health effects in occupational and environmental
settings. Because of its toxic potential, TiO$_2$ has been classified by the International Agency for Research on Cancer as “possibly carcinogenic to humans” by inhalation. Uptake of TiO$_2$ NPs can occur through multiple routes, including inhalation, ingestion and transdermal. Transdermal exposure of TiO$_2$ NPs is linked to the use of sunscreen and cosmetics, although there is no evidence demonstrating that TiO$_2$ can penetrate into normal skin. Actually, the major route of human exposure to TiO$_2$ NPs is through its use as a pharmaceutical additive and through food intake, where TiO$_2$ has been widely used as a coloring agent for the food industry. Additionally, studies dealing with oral exposure of TiO$_2$ NPs in mice have demonstrated the presence of particles in distant organs such as the liver, spleen, kidneys and lungs. These data suggest that TiO$_2$ particles can travel to other tissues and organs following uptake by the gastrointestinal tract, with blood circulation primarily implicated in its biodistribution. A number of studies for TiO$_2$ toxicity in animals have focused on inhalation although potential inhalation exposure to TiO$_2$ NPs occurs mostly in the workplace. Because of the physicochemical similarities between ambient, nanoscale particles and NPs, there is a strong rationale linking exposure to TiO$_2$ NPs with adverse cardiovascular effects. Past studies have demonstrated that inhaled ambient ultrafine particles can reach deep into the lungs where they can enter the circulatory system, resulting in cardiovascular diseases. Also, multiple studies reporting epidemiological animal data have established a link between ambient particles and the etiology of cardiovascular disease. Other exposure routes include surgical implant-derived wear debris and intravenously administered contrast agents. Although intravenous exposure cases are rare, there is potential concern because a direct injection of TiO$_2$-containing contrast agents into the circulatory system may confer a greater impact on the vascular endothelium due to near-100% bioavailability.

Vascular endothelial cells are potential targets for TiO$_2$ NP toxicity in human exposure. There are several cellular events responsible for the initiation of atherosclerosis in the vascular endothelium, including oxidative stress, inflammation and activation of endothelial cells. Previous studies have evaluated endothelial activation and dysfunction in endothelial cells but the intracellular signaling pathways are not fully identified. The purpose of this study is to determine the intracellular signaling pathways by which TiO$_2$ NPs induce inflammatory responses in vascular endothelial cells. Our data demonstrate that TiO$_2$ NPs increase vascular adhesion molecules such as monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1.
(VCAM-1), and that this is mediated by multiple intracellular signaling pathways including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 kinase (PI3K)/Akt.

3.A.3 Materials and Methods

3.A.3.1 Materials

Commercial grade uncoated TiO$_2$ NPs (anatase, metal basis, stock# 44689, 99.9% purity, 5 nm) were purchased from Alfa Aesar (Ward Hill, MA). DMSO, apocynin, PD98059, SB203580, SP600125 and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO). The NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) was obtained from Calbiochem (Darmstadt, Germany). Epigallocatechin gallate (EGCG) was purchased from Cayman Chemical (Ann Arbor, MI). VCAM-1, IκBα and p-IκBα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for extracellular signal-regulated kinase-1/2 (ERK1/2), p42/44, p38 MAPK, c-Jun-N-terminal kinase (JNK) and Akt were obtained from Cell Signaling Technology (Danvers, MA). LC3 antibody was purchased from Novus Biologicals (Littleton, CO).

3.A.3.2 Nanoparticle characterization

TiO$_2$ nanoparticle size, surface area and purity were evaluated in comparison to vendor specifications. Elemental analysis of dry TiO$_2$ samples was performed using a Hitachi S-4300 scanning electron microscope (Dallas, TX) and Princeton Gamma-Tech energy dispersive X-ray spectroscopy (EDS) Microanalysis System (Princeton, NJ), primary and aggregate particle sizes were determined using scanning electron microscopy (SEM), and surface area was determined through the Brunauer-Emmett-Teller (BET) method using a Micromeritics TriStar 3000 surface area and pore size analyzer (Norcross, GA), following overnight nitrogen degassing at 120 ºC. Characterization results can be found in Table 3.A.1 as compared to vendor-supplied values. Suspensions of TiO$_2$ NPs in cell culture media were prepared at a concentration of 5 mg/mL. Nanoparticle dispersion and size characteristics were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 particle size analyzer (Malvern Instruments, Westborough, MA), which measures hydrodynamic diameters of nanoparticles$^{209}$. TiO$_2$ NP suspensions (n=3) were suspended in deionized H$_2$O, sonicated briefly using a probe sonicator (Heat Systems, Inc., Farmingdale, NY) for 15 min to minimize particle aggregates, added to cell culture medium and analyzed via DLS.
with five measurements per sample. The same dispersion procedure was also used prior to in vitro studies. Sizing data, including mean nanoparticle size (nm) and particle size ranges, was determined using Malvern DTS Software, v. 6.32 (Table 3.A.1).

3.A.3.3 Primary cell culture and endothelial cell treatments

Primary vascular endothelial cells were isolated from porcine pulmonary arteries and characterized as described previously\textsuperscript{77, 210}. Cells were cultured in M199 media (Gibco, Grant Island, NY) supplemented with 10\% fetal bovine serum (FBS) (Invitrogen, Carlsbad). Cells were grown to confluence and serum starved overnight in medium containing 1\% FBS prior to initiation of cell treatments. A stock suspension of 5 mg/mL TiO\textsubscript{2} NPs was prepared and dispersed by probe sonication for 15 min. Based on our preliminary studies, we chose to treat cells with TiO\textsubscript{2} NPs at 10 and 50 µg/mL, which corresponded to 2 and 10 µg nanoparticles/cm\textsuperscript{2}, respectively. Of particular relevance to the present study, TiO\textsubscript{2} NPs have been suggested for use in intravenous applications as contrast agents\textsuperscript{205, 206}. Due to near-100\% bioavailability, potential intravenous applications could allow nanoparticles to achieve significantly higher concentrations in the blood circulation than that from translocation of nanoparticles following occupational and environmental exposure. These nanoparticle concentrations (10 and 50 µg/mL) were selected not only to address potential intravenous and environmental exposure levels but also to correspond with previous studies showing increased expression of inflammatory genes without cell death\textsuperscript{208, 211}. Equal volumes of water (up to 1\% of media; no hypotonic conditions were produced as shown by autophagy analysis in Figure 3.A.6) were used in place of NP-suspension volumes to serve as controls in cell culture. The TiO\textsubscript{2} NP concentrations and treatment intervals employed in these studies did not lead to significant cytotoxicity, as seen by trypan blue exclusion staining (data not shown).

3.A.3.4 Assessment of superoxide ($O_2^-$) levels

Endothelial cells were grown to confluence in 8-chamber culture slides (BD Biosciences, Bedford, MA). Following treatment, cells were incubated with a final concentration of 5 μM dihydroethidium (DHE), MitoSOX\textsuperscript{TM} Red mitochondrial superoxide indicator (MitoSOX) or DMSO (blank) in a 5\% CO\textsubscript{2} incubator for 15 min. Cells were washed 3x with PBS, fixed with 4\% formaldehyde, and washed again 3x with PBS. Slides were mounted with ProLong Gold Antifade reagent containing 4’6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) to visualize the nuclei. Slides were
evaluated under a Nikon ECLIPSE TE2000-U fluorescence microscope and the images were captured digitally using a Nikon LH-M100CB-1 camera and NIS-Elements BR 4.00.08 software (Nikon Instruments Inc. Melville, NY).

3.A.3.5 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of endothelial cells were prepared using NE-PER nuclear extraction reagents (Thermo, Rockford, IL). The concentration of nuclear extract was determined using Bradford reagent (Bio-Rad, Richmond, CA). DNA binding activities of NFκB were determined using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. DNA-binding reactions were performed with a final volume of 20 µL buffer containing 5 µg of nuclear extract, 50 ng/µL Poly (dl·dC) and biotin end-labeled oligonucleotides. Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The oligonucleotides containing the NFκB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were described previously[22]. EMSA gels were quantified by Image J software (NIH, Bethesda, MD).

3.A.3.6 Quantitative real-time PCR

Cells were grown in 6-well plates, and total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). The levels of mRNA expression were then assessed by quantitative real-time PCR using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems). Data analysis was performed using the relative quantification method (ΔΔCt), in which relative mRNA expression for target mRNAs (i.e., VCAM-1 and MCP-1) was compared to a constitutively expressed gene (i.e., β-actin) in the mRNA samples from untreated and treated cells. Primer sequences for SYBR Green chemistry were designed using the Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences for porcine VCAM-1, MCP-1 and β-actin were described in previous articles published by our laboratory[60].
3.A.3.7 Western blot analyses

Protein expression was determined using Western blot. Whole cells were lysed in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease and phosphatase inhibitor cocktails (Thermo, Waltham, MA). Lysed cells were centrifuged at 12,000 x g for 10 min at 4 °C. Protein levels of the supernatants were determined via BCA assay (Pierce, Rockford, IL). Protein samples (30 µg per treatment) were separated using 10% SDS-PAGE and subsequently were transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk buffer and incubated overnight at 4 °C with primary antibodies. After washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and visualized using ECL detection reagents (Thermo, Waltham, MA).

3.A.3.8 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way or two-way ANOVA followed by Student-Newman-Keuls method using Sigma Stat 3.1 software (Systat Software, San Jose, CA). A probability value $p < 0.05$ was considered statistically significant.

3.A.4 Results

3.A.4.1 Characterization of TiO$_2$ nanoparticles

Physicochemical characteristics of TiO$_2$ NPs are described in Table 3.A.1. TiO$_2$ size analysis using SEM showed average NP and aggregate diameters of 79 nm, ranging from 7 to 232 nm. BET analysis of TiO$_2$ NPs showed a specific surface area of 93.9 m$^2$/g. DLS particle size analysis in cell culture media showed particle aggregates 349 nm in diameter on average, with a range of 250 nm to 396 nm. EDS elemental analysis showed 99.7% TiO$_2$ NP purity, in keeping with manufacturer product information.

3.A.4.2 TiO$_2$ NPs increase superoxide production

Oxidative stress is a critical event of endothelial inflammation, activation and dysfunction. The fluorescent dye DHE is sensitive to reactive oxygen species and, in particular, to the superoxide anion. Once this dye is oxidized by superoxide, it stains cells a bright fluorescent red. Our results showed that TiO$_2$ NPs at concentrations of 10-50 µg/mL significantly increased superoxide generation at 1 h post exposure (Figure
3. A. 1). In addition, TiO$_2$ NP-induced superoxide production was confirmed by MitoSOX Red fluorescent dye which selectively detects mitochondrial superoxide production. Like DHE staining, 10-50 µg/mL of TiO$_2$ NP exposure markedly increased mitochondrial superoxide production (Figure 3.A.1). Superoxide production was not detected in cells treated with TiO$_2$ NPs (10-50 µg/mL) alone (Figure 3.A.1). No detectable fluorescence was seen in controls with TiO$_2$ NPs and fluorescent dye but without cells (data not shown).

3.A.4.3 Akt and three MAPK pathways are activated by TiO$_2$ NPs in endothelial cells

Oxidative stressors are known to activate many intracellular signaling pathways. In order to identity intracellular signaling pathways associated with oxidative stress and subsequent gene expression, we observed the phosphorylation of some key signaling molecules. The PI3K/Akt and MAPK signaling pathways are involved in a wide range of cellular processes, including NFκB activation, which subsequently can induce cell inflammation in response to extracellular stimuli$^{212, 213}$. We examined potential involvement of the MAPK signaling pathways (i.e., p38, ERK and JNK) in endothelial cells after treatment with various concentrations of TiO$_2$ NPs. Phosphorylation of endogenous p38, ERK and JNK was determined by Western blot analysis. Treatment with both 10 and 50 µg/mL TiO$_2$ NPs markedly increased all three MAPK pathways (Figure 3.A.2). Similar to activation of MAPKs, Akt was phosphorylated at Ser473 by TiO$_2$ NPs (Figure 3.A.2). Phosphorylation of Akt, JNK and ERK peaked as early as 30 min post-TiO$_2$ NP treatment. Phosphorylation of p38 reached a maximum at 1 h post-TiO$_2$ NP exposure.

3.A.4.4 TiO$_2$ NPs activate NFκB

Activation of three MAPKs and PI3K/Akt can lead to activation of the redox-sensitive transcription factor NFκB that subsequently up-regulates genes including VCAM-1 and MCP-1. Our EMSA results showed that TiO$_2$ NPs significantly increased NFκB DNA binding activity in vascular endothelial cells (Figure 3.A.3.A). Competition assays were conducted to ensure the specificity of NFκB DNA binding (Figure 3.A.3.A). Activation of NFκB was confirmed by phosphorylation of IκBα (Figure. 3.A.3.B).
3.A.4.5 TiO$_2$ nanoparticles increase gene and protein expression of MCP-1 and VCAM-1

Vascular endothelial cells were treated with TiO$_2$ NPs at concentrations of 0, 10, and 50 µg/mL for 2, 4, 8 and 16 h to determine TiO$_2$ NP-induced expression of inflammatory molecules such as MCP-1 and VCAM-1. TiO$_2$ NPs significantly induced expression of MCP-1 mRNA for all exposure times at a concentration of 50 µg/mL, compared to control (Figure 3.A.4.A). In contrast, MCP-1 mRNA was significantly increased only after a 2 h exposure at a concentration of 10 µg/mL TiO$_2$ NPs. Similarly, VCAM-1 mRNA expression was significantly up-regulated after cells were treated with TiO$_2$ NPs (50 µg/mL) for all exposure time points, while TiO$_2$ NPs (at 10 µg/mL) significantly increased VCAM-1 mRNA only at 2 h (Figure 3.A.4.B). Further, Western blot analysis showed that VCAM-1 protein was markedly increased when cells were treated with TiO$_2$ NPs (10 and 50 µg/mL) for 4 h (Figure 3.A.5). TiO$_2$ NPs at 50 µg/mL maintained significantly higher VCAM-1 protein level up to 16 h. To ensure that the observed inflammatory responses were derived from the NPs and not from surface impurities, PBS-washed TiO$_2$ NPs (using centrifugation, 3000 x g, for 5 min, 3 times) were compared with unwashed TiO$_2$ NPs for VCAM-1 and MCP-1 gene expression. The results showed similar proinflammatory effects by PBS-washed NPs compared to unwashed TiO$_2$ NPs (data not shown).

3.A.4.6 TiO$_2$ nanoparticles increase autophagy in endothelial cells

Protein expression of LC3-I and LC3-II was analyzed in order to determine if TiO$_2$ NPs and/or suspension medium increase autophagy levels in porcine endothelial cells. Our data showed that the addition of water (up to 1%) as the nanoparticle suspension medium did not lead to increased endothelial cell autophagy (Figure 2.A.6.A). Autophagy was markedly increased, though, when cells were treated with TiO$_2$ NPs (50 µg/mL), where a maximum expression of LC3-II was observed after an 8 h exposure (Figure 3.A.6.A). Due to the large surface area associated with NPs, nutrients can adsorb onto NPs in high quantities, which can potentially lead to nutritional deprivation and ultimately to increased autophagy. To address this, we tested if different concentrations of FBS (0, 1 and 5%) in TiO$_2$ suspension media can induce varying levels of autophagy in our cell culture model. A similar level of autophagy was observed only in cells treated with 50 µg/mL TiO$_2$ NPs while other treatment groups did not display autophagy, suggesting nutritional deprivation was not great enough to induce autophagy in our cell culture.
model (Figure 3.A.6.B). Comparison of water and PBS as NP-suspension media revealed no significant difference in the induction of autophagy (Figure 3.A.6.C).

3.A.4.7 Pharmacological inhibition of NFκB, oxidative stress, PI3K/Akt and MAPK pathways result in the decreased expression of MCP-1 and VCAM-1

In an effort to further identify signaling pathways associated with TiO₂ NP-induced endothelial inflammatory responses, we determined expression of adhesion molecules i.e., MCP-1 and VCAM-1 in cells pretreated with pharmacological inhibitors for three MAPKs, Akt, NFκB and oxidative stress. Pharmacological inhibitors such as PD98059, SB203580, SP600125 and LY294002 block phosphorylation of ERK, p38, JNK and Akt, respectively. Pretreatment of cells with these inhibitors significantly attenuated TiO₂ (10 µg/mL)-induced MCP-1 and VCAM-1 gene expression (Figure 3.A.7.A and 3.A.7.B), suggesting that all of these signaling molecules can mediate TiO₂-induced inflammatory pathways in vascular endothelial cells. Pretreatment of cells with the NFκB inhibitor PDTC for 30 min followed by TiO₂ treatment for 2 h resulted in a significant attenuation of MCP-1 and VCAM-1 gene expression, compare to TiO₂ NPs alone (Figure 3.A.7.A and 3.A.7.B), confirming that NFκB signaling is a critical mediator of these proinflammatory responses. Similarly, the antioxidant polyphenol EGCG and apocynin significantly down-regulated TiO₂ NP-induced expression of these genes (Figure 3.A.7.A and 3.A.7.B). Treatment with SP600125 (40 µM), LY294002 (40 µM) and apocynin (1 mM) significantly decreased base line MCP-1 mRNA expression but not in VCAM-1 expression. Overall, these data demonstrate that TiO₂ NP-induced inflammatory gene expression is mediated by oxidative stress and increased DNA binding of NFκB.

3.A.5 Discussion

Engineered nanoparticles have been suggested to increase the risk and incidence of cardiovascular diseases, including atherosclerosis. Occupational and environmental exposure of TiO₂ NPs may be a risk factor for increased cardiovascular inflammatory responses in humans. Aerosolized sunscreens may lead to higher inhalation absorption and subsequent translocation of TiO₂ NPs into the circulatory system. Also, intravenous exposure of TiO₂ NPs for biomedical uses is of special concern due to their higher bioavailability in the human vasculature.
As seen in the current studies and also observed by others, anatase TiO$_2$ NPs tended to agglomerate to sizes ranging from 250 nm to 396 nm in diameter in cell culture media containing 1% fetal bovine serum. In cultured endothelial cells, TiO$_2$ NPs and/or TiO$_2$ NP agglomerates were internalized by cells within one hour, and visualized using a relatively simple fluorescent microscopy method using ARS that labels TiO$_2$ NPs\textsuperscript{215}. A recent study also highlighted the cellular uptake of TiO$_2$ NPs by cells where nanoparticles were massively internalized into cytoplasmic vacuoles in endothelial cells\textsuperscript{216}; these findings were confirmed using transmission electron microscopy. These previous reports suggest that endothelial cells can internalize both agglomerated and non-agglomerated nanoparticles, although the underlying mechanism associated with nanoparticle uptake is not fully identified.

In the present study, we tested expression of adhesion molecules, particularly VCAM-1 in primary vascular endothelial cells, when treated with anatase TiO$_2$ NPs. The role of endothelial inflammation and expression of adhesion molecules such as MCP-1 and VCAM-1 in early atherosclerotic lesion formation is critical, and studies have found that by blocking VCAM-1 expression, one can reduce monocyte adhesion by 75%\textsuperscript{217}. Results from the current study demonstrated that MCP-1 and VCAM-1 gene expression was significantly higher in cells exposed to TiO$_2$ NPs. Both 10 and 50 µg/mL of TiO$_2$ NPs exhibited the highest gene expression levels after a 2 h exposure. Both MCP-1 and VCAM-1 gene expression were diminished for longer times points when cells were treated with 10 µg/mL of TiO$_2$ NPs, while 50 µg/mL of TiO$_2$ NPs sustained the elevated gene expression up to 16 h. Many mechanisms may be responsible for the decrease of gene expression during the extended exposure time. A recent study demonstrated that TiO$_2$ NPs elicit oxidative damage that can lead to the activation of nuclear factor erythroid 2 related factor 2 (Nrf2) as a protective mechanism\textsuperscript{218}. Nrf2-regulated genes include many antioxidant proteins such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S transferase (GST). Enhanced expression of these antioxidant proteins resulted in the reduction of VCAM-1 and MCP-1 gene expression in endothelial cells\textsuperscript{60}. In the present study, the cellular defensive system is sufficient to reduce TiO$_2$ NP-induced inflammatory gene expression at a concentration of 10 µg/mL. In contrast, 50 µg/mL of TiO$_2$ NPs was able to overcome the cell’s defensive mechanisms, leading to extended inflammatory gene expression. VCAM-1 protein expression showed a similar pattern to that of mRNA but the maximum expression was observed at 4 h. These data
show a potential trend indicating concentration- and time-dependent inflammatory effects associated with TiO₂ NP exposure.

Autophagy is a cellular process involving the lysosomal degradation of cytoplasmic components and is essential for cell survival and homeostasis²¹⁹. Our data showed that autophagy was increased in cells exposed to 50 µg/mL TiO₂ NPs while treatment with 10 µg/mL TiO₂ NPs did not increase autophagy. Multiple forms of cellular stress can induce this cellular defensive process and TiO₂ NPs additionally were found to increase autophagy in cells²²⁰;²²¹. Autophagy is a complex process which is linked to other stress responses. Recent studies have demonstrated that autophagy can negatively regulate NFκB signaling and gene expression such as MCP-1²²²-²²⁴. In our study, however, MCP-1 and VCAM-1 gene expression was not significantly affected by autophagy, although limited decrease in expression of these genes was seen 8 h post TiO₂ exposure, which occurs concurrently with maximal induction of autophagy. With our current data, it is not clear if TiO₂ NP-induced autophagy is a significant factor for the observed gene expression, therefore further studies are needed to elucidate the underlying mechanism.

We also found that TiO₂ NPs can increase cardiovascular inflammation by activation of NFκB and subsequent up-regulation of endothelial inflammatory parameters, such as MCP-1 and VCAM-1. Further, mechanisms and signaling pathways associated with TiO₂ NP-induced endothelial cell toxicity were studied. TiO₂ NPs stimulated production of ROS (e.g., superoxide), which activated four key signaling molecules: three MAPKs (p38, ERK and JNK) and Akt. Activation of such signaling molecules and expression of endothelial inflammatory parameters were dependent on the concentration of TiO₂ NPs (0-50 µg/mL). As stated previously, induction of adhesion molecules including VCAM-1 and MCP-1 is the product of NFκB activation²²⁵;²²⁶; NFκB is a critical redox-sensitive transcription factor by which expression of inflammatory genes is regulated by extracellular stimuli in cells²²⁷;²²⁸. In the present study, we found that TiO₂ NPs can activate NFκB, which is demonstrated by increased DNA binding activity of NFκB and increased phosphorylation of IκBα. These results subsequently were confirmed in cells pretreated with a specific NFκB inhibitor (i.e., PDTC) where this inhibitor served to significantly attenuate TiO₂ NP-induced VCAM-1 and MCP-1 gene expression. Three MAPK proteins and Akt signaling protein are known to be associated with activation of NFκB²²⁷. Cellular oxidative stress is closely associated with
phosphorylation of such signaling molecules and subsequent NFκB activation. Recent studies have demonstrated that ROS are produced by TiO$_2$ NPs and play an inflammatory role in cells\cite{220}. Another recent study also implicated ROS in the activation of NFκB and subsequent induction of inflammatory genes in human endothelial cells treated with silica nanoparticles\cite{229}. Similarly, results in the current study demonstrated that TiO$_2$ NPs induce ROS production; our data show a significant production of superoxide anion after cell exposure to TiO$_2$ NPs. Further, in order to confirm these data, we pretreated cells with antioxidants such as EGCG and apocynin, which resulted in a significant decrease in VCAM-1 and MCP-1 inflammatory gene expression when compared to treatment with TiO$_2$ NPs alone. Mechanisms responsible for TiO$_2$ NP-induced ROS production are not well defined but possibly are linked to the activation of NADPH oxidase (NOX) because apocynin, a specific inhibitor for NOX activation, can attenuate TiO$_2$ NP-induced gene expression for MCP-1 and VCAM-1\cite{230}. These results suggest that TiO$_2$ NPs can induce ROS production and that this is a key mechanism associated with TiO$_2$ NP-induced MAPKs, Akt and NFκB activation.

The mechanisms associated with TiO$_2$ NP-induced inflammation are not simple and likely involve activation of many intracellular signaling molecules. In our studies with endothelial cells, treatment with TiO$_2$ NPs phosphorylated three MAPKs and PI3K/Akt. Phosphorylation of these key signaling molecules can lead to the activation of redox-sensitive transcription factors, such as NFκB\cite{227}. Indeed, TiO$_2$ NPs increased phosphorylation of three MAPKs and PI3K/Akt, and DNA binding of NFκB subsequently led to induction of MCP-1 and VCAM-1. The roles of these three MAPKs and PI3K/Akt were confirmed by pretreating cells with specific MAPK and PI3K/Akt inhibitors, which resulted in a significant decrease in VCAM-1 and MCP-1 gene expression compared to treatment with TiO$_2$ NPs alone. These data suggest a specific role of intracellular signaling pathways in endothelial inflammation following TiO$_2$ NP exposure.

Taken together, data suggest that TiO$_2$ NPs used in our study can increase inflammatory gene expression in vascular endothelial cells via redox-sensitive signaling pathways. This work highlights the toxic effects of anatase TiO$_2$ NPs in the cardiovascular system to better understand potential overall public health concerns associated with metal oxide nanoparticles. (The data presented in Chapter 3.A were published\cite{39}.)

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<th>Vendor</th>
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<th>Vendor Surface Area (m²/g)</th>
<th>Analyzed SEM Avg Size and (Size Range) (nm)</th>
<th>Analyzed DLS Avg Aggregate Size and (Size Range) (nm)</th>
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Figure 3.A.1. TiO$_2$ NPs increase superoxide production in endothelial cells. Endothelial cells grown on 8-chamber culture slides were treated with TiO$_2$ NPs for 2 h. Cells were then stained with either DHE or MitoSOX, and the intensity of red fluorescence was assessed using a fluorescence microscope. The red areas in the cells represent oxidized DHE and MitoSOX representing the generation of superoxide. The nuclei were stained with DAPI. The images shown here are representatives of three independent experiments.
Figure 3.A.2. TiO$_2$ NP-induced phosphorylation of MAPKs and PI3K/Akt pathways in endothelial cells. Cells were treated with TiO$_2$ NPs to observe the activation of three types of MAPK signaling pathways, p38, ERK and JNK, as well as the Akt signaling pathway. The images shown here are phosphorylated signaling molecules at the time of maximum induction. The Western blots shown here are representative images of three independent experiments.
Figure 3.A.3. TiO$_2$ NPs increase activation of NFκB. (A) NFκB DNA binding determined by EMSA analysis. (B) Phosphorylation of IκBα analyzed in whole cell lysate by Western blot. Cells were treated with TiO$_2$ NPs (0-50 µg/mL) for 2 h followed by preparation of nuclear extracts for EMSA or whole cell lysates for Western blot. In EMSA, unlabeled competitor sequences were present at a 200-fold molar excess over labeled sequences. Data represent the mean±SEM, with n=3. Experiments were repeated a minimum of three times. The EMSA and Western blot shown here is a representative image.

*Significantly different compared to control (p<0.05).
Figure 3.A.4. TiO$_2$ NPs increase expression of MCP-1 (A) and VCAM-1 (B) genes. Gene expression levels were measured using quantitative real-time PCR. Primary endothelial cells were treated with 0-50 µg/mL of TiO$_2$ NPs for the indicated times (2-16 h). Results represent the mean±SEM, with n=3. Experiments were repeated a minimum of three times. *Significantly different compared to control (p<0.05).
Figure 3.A.5. TiO$_2$ NPs increase VCAM-1 protein expression in primary endothelial cells. Primary endothelial cells were treated with 0-50 µg/mL of TiO$_2$ NPs for different time points (2-16 h). Western blots show a dose-dependent increase of VCAM-1 by TiO$_2$ NPs. Western blots shown here are representative images of three independent blots.
Figure 3.A.6. Expression of LC3-I/II, an autophagy marker, by endothelial cells. (A) Endothelial cells were treated with 0-50 µg/mL TiO$_2$ NPs at different time points (2-16 h). (B) Endothelial cells were treated with 0-50 µg/mL TiO$_2$ NPs for 8 h using three different NP suspension media: water only, water with 1% FBS and water with 5% FBS. (C) Endothelial cells were treated with 0-50 µg/mL TiO$_2$ NPs for 8 h using two different NP suspension media: water and PBS. Western blots shown here are representative images of three independent blots.
Figure 3.A.7. Pharmacological inhibition of NFκB, oxidative stress, PI3K/Akt and MAPK pathways result in the decreased expression of MCP-1 and VCAM-1. Expression of MCP-1 (A) and VCAM-1 (B) mRNA in endothelial cells. Cells were exposed to TiO₂ NPs (10 µg/mL) for 2 h after a 30 min pretreatment with pharmacological inhibitors, i.e., PD98059 (40 µM, ERK inhibitor), SB203580 (40 µM, p38 inhibitor), SP600125 (40 µM, JNK inhibitor) and LY294002 (40 µM, Akt inhibitor). Also, cells were pretreated with the NFκB inhibitor (PDTC, 10 µM, for 1 h) and antioxidants (EGCG, 50 µM for 3 h and apocynin, 1 mM for 1 h) followed by TiO₂ NP (10 µg/mL) exposure for 2 h. mRNA
expression levels were measured using quantitative real-time PCR. Results represent the mean±SEM, with n=3. Different letters correspond to significant differences (p<0.05) between treatment groups.
Chapter 3.B. PCB 77 dechlorination products modulate pro-inflammatory events in vascular endothelial cells

3.B.1 Synopsis

Persistent organic pollutants such as polychlorinated biphenyls (PCBs) are associated with detrimental health outcomes including cardiovascular diseases. Remediation of these compounds is a critical component of environmental policy. Although remediation efforts aim to completely remove toxicants, little is known about the effects of potential remediation byproducts. We previously published that Fe/Pd nanoparticles effectively dechlorinate PCB 77 to biphenyl, thus eliminating PCB-induced endothelial dysfunction using primary vascular endothelial cells. Herein, we analyzed the toxic effects of PCB congener mixtures (representative mixtures of commercial PCBs based on previous dechlorination data) produced at multiple time points during the dechlorination of PCB 77 to biphenyl. Compared to pure PCB 77, exposing endothelial cells to lower chlorinated PCB byproducts led to improved cellular viability, decreased superoxide production, and decreased nuclear factor kappa B (NFκB) activation based on duration of remediation. Presence of the parent compound, PCB 77, led to significant increases in mRNA and protein inflammatory marker expression. These data implicate that PCB dechlorination reduces biological toxicity to vascular endothelial cells.

3.B.2 Introduction

Polychlorinated biphenyls (PCBs) are a class of organic pollutants that were used heavily as dielectric fluids in industrial and electrical applications until evidence surfaced linking PCBs to cancer and cardiovascular disease. Remediation efforts for these highly persistent pollutants have proven difficult, expensive and slow. Our laboratories have developed new fast, low-cost techniques that employ Pd/Fe nanoparticles for reductive PCB dechlorination, which systematically dechlorinate higher congener PCBs to biphenyl, with lower chlorinated congeners produced as byproducts prior to complete degradation. The goal of this work is to better understand the toxicological effects of these byproducts and to further validate the importance of PCB dechlorination for reducing cardiovascular risk factors and for improving overall public health.
Even though PCB production has been banned in the US since the late 1970s, PCBs remain concentrated in many waterways and Superfund sites, as well as in electrical devices such as transformers and light ballasts. Additionally, recent research has indicated that a variety of PCB congeners are still being generated as industrial byproducts from pigment production and certain paper manufacturing\textsuperscript{231; 232}. PCBs exhibit unique properties, such as high environmental persistence and resistance to metabolism in organisms, which lead to difficulties for remediation. Additionally, the costs associated with large-scale clean-up and removal of these and other chemical pollutants is great, and thus the development of more efficient, cost effective technologies continues to be important. Current remediation techniques rely on expensive dredging followed by incineration or bioremediation techniques. Although PCBs can be degraded through some natural processes such as bacterial degradation\textsuperscript{233; 234} and photolysis\textsuperscript{235; 236}, these processes are prohibitively slow for remediation of larger scale contaminations. Our research group recently developed a Pd/Fe nanoparticle system that catalyzes the dechlorination of PCBs to form biphenyl in order to address this need for low cost, high throughput chlorinated aromatic degradation\textsuperscript{237; 238}. During chlorinated aromatic degradation, hydrogen ions are used to displace chlorine on biphenyl rings in stages, resulting in the production of lower chlorinated PCB congeners prior to complete degradation to biphenyl. It is generally believed that less-chlorinated congeners are more water soluble, more volatile, and more likely to biodegrade further\textsuperscript{239}. In a previous study, we compared the biological activity of the parent compound (PCB 77) with the dechlorination end product (biphenyl), and found that complete dechlorination markedly reduced the pro-inflammatory activity of PCB 77\textsuperscript{240}.

Many environmental contaminants, and especially persistent organic pollutants, are risk factors for cardiovascular diseases such as atherosclerosis because they can initiate or exacerbate the underlying disease by altering gene expression patterns and subsequent vascular inflammation\textsuperscript{57; 241}. Because the endothelium is in immediate contact with the blood, endothelial cells are particularly susceptible to the effect of environmental contaminants. The lining of blood vessels is protected by the endothelium, and endothelial cells play an active role in physiological processes such as regulation of vessel tone, blood coagulation, and vascular permeability. In turn, endothelial cell activation or dysfunction is a critical marker of the pathology of cardiovascular diseases such as atherosclerosis.
Activated endothelial cells produce chemokines such as monocyte chemoattractant protein 1 (MCP-1) and inflammatory cytokines, which attract monocytes from the bloodstream to the site of injury. Part of the resulting inflammatory process includes the endothelial cell expression of adhesion molecules, like vascular adhesion molecule-1 (VCAM-1), which allow the monocytes to attach to the endothelial layer and infiltrate this barrier into the intimal space. We have demonstrated previously that coplanar PCBs (e.g., PCB 77) can induce DNA-binding activity of the oxidative stress-sensitive transcription factor nuclear factor kappa B (NFкB) and expression of VCAM-1, which is dependent on functional aryl hydrocarbon receptor (AhR) activity. Recently, we have reported the mechanisms of PCB 77-mediated up-regulation of MCP-1.

Little is known about vascular toxicity of PCBs, and especially about the toxicity of dechlorination or remediation products. There is a clear need to evaluate products of remediation (e.g., dechlorination products) for biological activity in mammalian systems. It is important to know to what degree remediation processes of persistent organic pollutants have to occur before toxicity and any vascular injury becomes negligible. Thus, the present study was designed to test the effects of PCB 77 dechlorination products on pro-inflammatory parameters in a vascular endothelial cell model system. Data from this study suggest that the presence of the parent compound (e.g., PCB 77) is necessary for maximal endothelial cell dysfunction and inflammation. These data also suggest that dechlorination is a successful method for decreasing biological toxicity, with overall toxicity linked to the level of degradation of the parent compound. Herein, dechlorination is demonstrated to be an effective platform for addressing public health concerns associated with these persistent chlorinated pollutants.

3.B.3 Materials and Methods

3.B.3.1 Materials

Biphenyl, 3-chlorobiphenyl (PCB 2), 4-chlorobiphenyl (PCB 3), 3,3’-dichlorobiphenyl (PCB 11), 3,4-dichlorobiphenyl (PCB 12), 3,4’-dichlorobiphenyl (PCB 13), 4,4’-dichlorobiphenyl (PCB 15), 3, 3’,4-trichlorobiphenyl (PCB 35), 3,4,4’-trichlorobiphenyl (PCB 37) and 3, 3’, 4, 4’-tetrachlorobiphenyl (PCB 77) were purchased from AccuStandard, Inc. (New Haven, CT). Experiments that included hazardous materials such as PCBs were performed in accordance with institutional and federal
guidelines. All cell culture reagents, including ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI), were purchased from Life Technologies (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO), unless otherwise specified.

3.3.3 Description of PCB 77 dechlorination treatment mixtures

Treatment mixtures were based on the composition of PCB 77 and its dechlorination products at 0, 10, 24, and 48 hours of remediation as derived from GC-MS data comparing the reductive efficiencies of 0.1 mg/mL 400 nm Pd/Fe nanotubes and 1 mg/mL Pd/Fe nanoparticles. Dechlorination product compositions were determined from these data (Table 3.B.1); treatments from each stage of the dechlorination are described as percent by weight and as molarity. Stocks of individual PCB commercial congeners were solubilized in fresh dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Treatment mixtures were developed to delineate between the effects of the parent compound and dechlorination products including treatments representing product mixtures without PCB 77, treatments representing only the concentration of PCB 77 at a particular stage of dechlorination, and a treatment representing the 24 h dechlorination products with the higher PCB 77 concentration found following 10 h dechlorination, to emphasize the role of PCB 77 in overall toxicity (Table 3.B.2). Stock treatment mixtures were prepared in DMSO at a 5 mM total concentration and diluted 1:1000 in cell culture medium for 5 µM final cell culture treatments.

3.3.3 Cell culture

Primary endothelial cells (ECs) were isolated from porcine pulmonary arteries as described previously. Cell culture media consisted of medium 199 (M199) containing 10% fetal bovine serum (FBS). At 70-80% confluence, cells were incubated overnight with treatment media (M199 with 1% FBS), followed by exposure to PCB 77 or respective treatment mixtures for 24 h. Treatment mixtures were added to cell culture at a final concentration of 5 µM, which represented a maximum concentration of 0.1% v/v DMSO/media (see Tables 3.B.1 and 3.B.2 for PCB treatment concentrations). Cell viability was tested in the presence of dechlorination products using the Vybrant MTT Cell Proliferation Assay Kit (Invitrogen, Eugene, OR) according to manufacturer guidelines.
3.B.3.4 Assessment of superoxide (O$_2^-$) levels

Superoxide levels were assessed as recently reported$^{70}$. Cells were grown to confluence in 4-chamber culture slides (BD Biosciences, Bedford, MA). After 4 h exposure to PCB treatments (5 µM total concentrations; Table 3.B.1), the cells were rinsed 2x with Krebs-Ringer buffer (KRB) and incubated with 5 µM dihydroethidium (DHE) or KRB (blank) at 37°C for 30 min. Cells were rinsed with KRB, fixed with 10% buffered formalin, and washed with PBS. Slides were mounted with ProLong Gold antifade reagent with DAPI for nuclei staining. Slides were evaluated with an Olympus BX61W1 fluorescence microscope. Mean fluorescence intensity was quantified using ImageJ 1.42q (NIH, Bethesda, MD).

3.B.3.5 Assessment of NFκB activation

Nuclear extracts were prepared from endothelial cells cultured as described above in 10 cm dishes and treated with PCB 77 or dechlorination mixtures for 4 h. The nuclear extraction was performed as described previously$^{22}$. EMSA (electrophoretic mobility shift assay) of nuclear factor kappa B (NFκB) binding was kindly performed by Dr. Seong-Su Han of the University of Iowa College of Medicine using the DNA-protein binding detection kit (Gibco-BRL, Grand Island, NY) with radio-labeled oligonucleotides$^{60,77}$.

3.B.3.6 Measurement of CYP1A1, MCP-1 and VCAM-1 mRNA

CYP1A1, MCP-1 and VCAM-1 mRNA expression was assessed using real-time PCR (RT-PCR), as described previously$^{23,77}$. Briefly, mRNA was isolated using TRIzol Reagent (Life Technologies, Carlsbad, CA) and quantified using a SmartSpec Plus Spectrophotometer (BIO-RAD, Philadelphia, PA). cDNA was then generated using Promega AMV reverse transcriptase (Fisher Scientific, Waltham, MA) and RT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) and the following porcine primer sequences: β-actin sense 5'-TCA TCA CCA TCG GCA ACG-3' and antisense, 5'-TTC CTG ATG TCC ACG TCG-3'; CYP1A1 sense 5'- TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA GGC AAG AGT AGT TGG AGA ACG GAG TAG CTC ATA-3'; MCP-1 sense 5'-CGG CTG ATG AGC TAC AGA AGA GT-3'and antisense, 5'-GCT TGG GTT CTG CAC AGA TCT-3'; and VCAM-1 sense 5'-TGG AAA GAC ATG GCT GCC TAT-3' and antisense, 5'-ACA CCA CCC CAG TCA CCA TAT-3'. Assays
were run using the Applied Biosystems 7300 Real time PCR System (Life Technologies, Carlsbad, CA) using absolute quantification. The raw data were quantified using a standard curve and analyzed with a manual C politique {\textit{T}} threshold of 0.200. Sample data were normalized to individual \( \beta \)-actin values.

3.B.3.7 Measurement of CYP1A1, MCP-1 and VCAM-1 protein

CYP1A1 and VCAM-1 protein levels were assessed by Western blotting, as described previously\(^{240} \). After semi-dry transfer, nitrocellulose membranes were incubated for 2\,h in blocking buffer (5\% non-fat milk in Tris-buffered saline containing 0.05\% Tween 20). Rabbit polyclonal CYP1A1 (H-70) and VCAM1 (C-19)-R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies were diluted 1:1000 in blocking buffer and incubated overnight at 4 °C, and the secondary antibody was diluted 1:4000. \( \beta \)-actin primary and secondary antibodies were diluted 1:10,000\(^{23} \). MCP-1 protein levels were measured in cell culture media diluted 1:7 using an OptEIA Human MCP-1 ELISA Kit (BD Biosciences, San Diego, CA) according to manufacturer guidelines.

3.B.3.8 Statistical analysis

Values are reported as means \( \pm \) SEM obtained from a representative sample set of a minimum of three independent experiments, unless otherwise stated. Comparisons were made by one-way analysis of variance (ANOVA) with Tukeys test for post-hoc analysis, using SigmaPlot 12.0 software (Systat Software, San Jose, CA). Statistical probability of \( p \)-value < 0.05 was considered significant. Similar statistical marker letters between treatments indicate no statistical difference.

3.B.4 Results

3.B.4.1 Dechlorination alters cellular oxidative stress and viability

PCB-induced cellular dysfunction is characterized by increased oxidative stress and subsequent activation of inflammatory pathways including NFkB. The uncoupling of CYP1A1 leads to the generation of reactive oxygen species (ROS), such as superoxide. Endothelial cell monolayers were exposed to representative dechlorination product mixtures (Table 3.B.1) for 4\,h before ROS were visualized with DHE fluorescence (Figure 3.B.1.A). Strong staining was observed in cells exposed to 0\,h (pure PCB 77) and 10\,h dechlorination products, whereas mixtures representing longer periods of dechlorination showed decreased superoxide production. When DHE fluorescence was
quantified and normalized to DAPI nuclear staining, fluorescence from initial dechlorination mixtures (0 and 10 h of dechlorination) indicated higher ROS production which decreased to control levels with further dechlorination (differences between groups approached significance, p=0.052; See Figure 3.B.1.B). A similar trend was observed with regard to viability, where no changes were observed in cells treated with PCB vehicle (DMSO) and the 48 h dechlorination product, although cell viability was decreased in 0 h (parent compound, PCB 77), 10 h and 24 h dechlorination product treatments to 69±1%, 61±3%, 87±2% of control, respectively.

3.B.4.2 NFκB activation is attenuated by dechlorination

Data from an NFκB EMSA showed that cells treated for 4 h with the dechlorination products exhibited a prominent increase in NFκB activation when treated with the 0 h product, or pure PCB 77. Intermediate activation by the 10 and 24 h products was observed; NFκB:DNA binding for these treatments was significantly decreased compared to exposure to the 0 h product but remained increased relative to the vehicle control and biphenyl (48 h product; see Figure 3.B.2). Biphenyl was consistently within control expression levels for superoxide fluorescence, viability, and NFκB:DNA binding. The 10 and 24 h dechlorination products were increased from control but did not exhibit a consistent linear change from parent PCB 77, 0 h of dechlorination, expression levels. We, therefore, tested a series of treatment mixtures to delineate between the effects of the dechlorination products and the residual PCB 77.

3.B.4.3 Dechlorination products alter CYP1A1, MCP-1 and VCAM-1 mRNA expression

CYP1A1, VCAM-1 and MCP-1 levels were assessed in mRNA using treatment groups 1-10 (Figure 3.B.3, Table 3.B.2). CYP1A1 expression is a sensitive marker of coplanar PCB-mediated cellular activation and toxicity. Thus, significant response from all treatments containing PCB 77 was expected (Figure 3.B.3.A). Indeed, all treatments that contained pure or some PCB 77 (treatments 2, 3, 5, 7, 9 and 10) exhibited almost maximal CYP1A1 expression. There was no difference in CYP1A1 expression among the pure PCB 77 (treatment 2) and both 10 h (treatment 3) and 24 h (treatment 5) dechlorination mixtures. Interestingly, when 10 h and 24 h dechlorination treatments lacked PCB 77 (treatments 4 and 6, respectively), induction of CYP1A1 was reduced linearly. Both MCP-1 and VCAM-1 mRNA expression patterns (Figures 3.B.3.B and
3.B.3.C, respectively) followed approximately the CYP1A1 treatment patterns, suggesting some correlation between CYP1A1 induction and inflammatory markers in these dechlorination experiments. As with CYP1A1 expression, MCP-1 and VCAM-1 expression exhibited a linear decrease to control levels when treated with mixtures representing 10, 24 and 48 h dechlorination products without PCB 77 (treatments 4, 6 and 8), indicating that any byproduct contribution to the inflammatory response was eliminated by further dechlorination. Total dechlorination to biphenyl (48 h product) showed no statistical difference from control, indicating that it was relatively benign in our EC model. The presence of residual parent PCB dominated the inflammatory response in product mixtures, but mRNA expression levels of PCB 77 alone were moderately attenuated at the lower concentrations.

3.B.4.4 Degree of dechlorination affects CYP1A1, MCP-1 and VCAM-1 protein expression

Endothelial cells were treated with the dechlorination mixtures mentioned above (Table 3.B.2) in order to determine the effects of PCB 77 dechlorination on endothelial cell inflammatory response expressed in protein markers (Figure 3.B.4). Treatments containing the parent compound showed strong correlations in both mRNA and protein. Similar to the mRNA expression levels, CYP1A1 protein levels were highest for treatments containing PCB 77 (Figure 3.B.4.A). However, treatments that did not contain PCB 77 showed a marked decrease in CYP1A1 and MCP-1 protein expression, correlated to the degree of dechlorination. Significant increases in MCP-1 expression were observed with the 0 h product (PCB 77, treatment 2) and, to a lesser degree, with dechlorination products containing decreasing concentrations of PCB 77 (treatments 3, 5, and 8). VCAM-1 protein expression of dechlorination product mixtures without PCB 77 (treatments 4 and 6) and biphenyl (treatment 8) were not significantly different from control. The presence of PCB 77 in all treatment groups corresponded to significant increases in VCAM-1 protein expression above control levels. These data suggest a complicated interplay between inflammatory parameters in response to PCB 77 dechlorination product exposure. Nevertheless, the presence of PCB 77 in the individual treatments markedly induced vascular inflammation.
3.B.5 Discussion

PCBs are man-made chemicals that were used in hundreds of industrial and commercial applications\textsuperscript{244} and are byproducts of certain industries today\textsuperscript{232}. Even though their mass production has been banned, PCBs are chemically stable, persistent environmental toxicants that can cause harmful health effects. Remediation or detoxification of contaminated sites is difficult and expensive, and it is not clear to what extent these remediation processes have to occur before their disease potential is mitigated. The present study was designed to test the hypothesis that dechlorination of coplanar PCB 77 diminishes its pro-inflammatory potential in vascular endothelial cells. This is an important issue because PCBs can damage vascular tissues and thus contribute to cardiovascular diseases such as atherosclerosis. In fact, a recent study reported increased hospitalization rates for acute myocardial infarction and diabetes mellitus in populations residing near areas contaminated with persistent organic pollutants\textsuperscript{245; 246}. Furthermore, the administration of PCB 77 to male apolipoprotein E (ApoE) \textsuperscript{--} mice has been shown to promote atherosclerosis\textsuperscript{247}.

The lining of blood vessels is protected by the endothelium, and endothelial cells play an active role in physiological processes such as regulation of vessel tone, blood coagulation, and vascular permeability. Dysfunction of endothelial cells is a critical underlying cause of the initiation of cardiovascular diseases such as atherosclerosis. Coplanar PCBs are initiators of endothelial dysfunction and exert their toxicity through binding to the AhR. AhR target genes, including CYP1A1, are considered a source of oxidative stress in endothelial cells and subsequent vascular dysfunction\textsuperscript{248; 249}. We confirmed in the present study the oxidative stress potential of coplanar PCBs. In fact, CYP1A1 induction was maximal in cells exposed to pure PCB 77 and remained elevated in all dechlorination mixtures that contained PCB 77, independent of its relative concentration. When residual amounts of PCB 77 were excluded from the dechlorination mixtures, CYP1A1 induction was markedly reduced, suggesting that the presence of the parent coplanar PCB is necessary for maximal CYP1A1 expression. Furthermore, we have previously shown that coplanar PCBs 77, 126 and 169 increased expression of the CYP1A1 gene, oxidative stress (DCF fluorescence), and the DNA-binding activity of NF\textsubscript{κ}B\textsuperscript{20}. In the current study, oxidative stress and DNA-binding of NF\textsubscript{κ}B decreased with increasing dechlorination. In fact, when PCB 77 was completely dechlorinated to biphenyl, oxidative stress and NF\textsubscript{κ}B levels approached control levels.
This suggests that effective dechlorination should be as complete as possible to avoid oxidative stress-mediated tissue toxicity.

Oxidative stress-induced transcription factors such as NFκB, which regulate inflammatory cytokine and adhesion molecule production, play critical roles in the induction of inflammatory responses and subsequent atherosclerotic lesion formation. We have demonstrated previously that coplanar PCBs can cause endothelial cell dysfunction as determined by inflammatory markers, such as expression of inflammatory cytokines and adhesion molecules, i.e. MCP-1 and VCAM-1, respectively 23; 56; 77. In our analysis of the dechlorination product mixtures, we found that inflammatory patterns of MCP-1 and VCAM-1 were similar to CYP1A1 patterns induced by pure PCB 77 and the dechlorination mixtures that contained residual PCB 77, suggesting that the parent compound is necessary for an endothelial inflammatory response. The 10 h dechlorination byproducts also induced an MCP-1 and VCAM-1 response that was alleviated by further dechlorination. Again, as was found with CYP1A1, total dechlorination to biphenyl did not induce MCP-1 and VCAM-1 inflammatory parameters in our endothelial cell model system.

The dechlorination process decreased the parent PCB 77 but contributed to various lower-chlorinated PCBs. Furthermore, the ratio and proportion of these lower-chlorinated PCBs changes with degree of dechlorination. Even though their level of interaction with the AhR is not well characterized, lower-chlorinated PCBs may contribute to cellular dysfunction. For example, PCB 15, which was found in relatively high concentrations in the dechlorination mixtures, can contribute to liver carcinogenesis 250. PCB 11, another common PCB in our dechlorination mixtures, recently has been discovered in relatively large amounts in commercial paint pigments 232. Furthermore, other lower chlorinated PCBs generated during dechlorination have been shown to be metabolically activated by electrophilic quinoid species, which can bind to DNA 251. Our data suggest that the temporary formation of lower chlorinated PCBs during dechlorination can contribute in part to induction of inflammatory parameters.

In addition, the lipophilic nature of PCBs enables them to perturb membrane structures. We have evidence that membrane domains like caveolae are critically involved in endothelial inflammation induced by exposure to PCBs. For example, PCB 77 can accumulate in caveolae-rich fractions of endothelial cells 83, and up-regulation of
endothelial MCP-1 by PCB 77 is caveolin-1-dependent\textsuperscript{23}. Evidence from our laboratory implicates caveolae as a regulatory platform involved in endothelial activation and inflammation by environmental contaminants (Figure 3.B.5). As a regulatory platform, caveolae facilitate the interaction of PCBs with the AhR, which in turn promotes the up-regulation of CYP1A1, a phase I detoxifying enzyme. CYP1A1 metabolism of coplanar PCBs, like PCB 77, is inefficient, leading to protein uncoupling and release of superoxide, which promotes the formation of reactive oxygen species (ROS) in the cell\textsuperscript{18}. The cell responds to this change in redox status by activating the transcription factor NF\textsubscript{k}B, which regulates enzymes and inflammatory parameters associated with endothelial dysfunction and cell injury such as MCP1 and VCAM1\textsuperscript{252}. Further studies are needed to understand whether caveolae have differential selectivity for various PCB species such as those present in dechlorination mixtures and to determine if the interaction of these congeners with caveolae has potential to induce endothelial cell dysfunction.

In summary, our study provides evidence that dechlorination of highly chlorinated PCBs is beneficial for protecting the vasculature from oxidative stress-induced inflammation and subsequent pathologies like atherosclerosis. Highly chlorinated PCBs are persistent and cytotoxic and a significant risk factor to endothelial injury and associated vasculature pathologies. The pro-inflammatory potential of the dechlorination mixtures that we observed appears to depend largely on the presence of the parent compound (e.g., PCB 77). Clearly, cytotoxicity decreased relative to degree of dechlorination, and the final dechlorination product, biphenyl, was nontoxic in our endothelial cell model system. More studies are needed to understand threshold concentrations of dechlorination mixtures that are required to prevent or mitigate compromised health associated with exposure to environmentally toxic chlorinated biphenyls. (The data presented in Chapter 3.B were published\textsuperscript{21}.)
**Table 3.B.1**
Treatment mixtures representing PCB 77 dechlorination byproducts at various time points during dechlorination.

<table>
<thead>
<tr>
<th>Mixture components</th>
<th>DMSO (vehicle)</th>
<th>0 h wt% (µM)</th>
<th>10 h wt% (µM)</th>
<th>24 h wt% (µM)</th>
<th>48 h wt% (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 77</td>
<td>100% (5)</td>
<td>20% (0.7)</td>
<td>7% (0.2)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 37</td>
<td>0%</td>
<td>4% (0.2)</td>
<td>2% (0.1)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 35</td>
<td>0%</td>
<td>4% (0.2)</td>
<td>2% (0.1)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 15</td>
<td>0%</td>
<td>11% (0.5)</td>
<td>9% (0.4)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 13</td>
<td>0%</td>
<td>8% (0.3)</td>
<td>7% (0.3)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 12</td>
<td>0%</td>
<td>8% (0.3)</td>
<td>7% (0.3)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 11</td>
<td>0%</td>
<td>11% (0.5)</td>
<td>9% (0.4)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 03</td>
<td>0%</td>
<td>3% (0.1)</td>
<td>3% (0.1)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>PCB 02</td>
<td>0%</td>
<td>3% (0.1)</td>
<td>3% (0.1)</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Biphenyl</td>
<td>0%</td>
<td>30% (1.9)</td>
<td>50% (3.1)</td>
<td>100% (5)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100% (5)</td>
<td>100% (5)</td>
<td>100% (5)</td>
<td>100% (5)</td>
<td></td>
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### Table 3.B.2

**Original and modified treatment conditions based on PCB 77 dechlorination byproduct**

<table>
<thead>
<tr>
<th>Mixture components</th>
<th>DMSO&lt;sup&gt;a&lt;/sup&gt; (0.1 %v/v vehicle)</th>
<th>0 h&lt;sup&gt;b&lt;/sup&gt; [µM (ppm)]</th>
<th>10 h&lt;sup&gt;b&lt;/sup&gt; [µM (ppm)]</th>
<th>10 h without PCB 77&lt;sup&gt;c&lt;/sup&gt; [µM (ppm)]</th>
<th>24 h&lt;sup&gt;b&lt;/sup&gt; without PCB 77&lt;sup&gt;c&lt;/sup&gt; [µM (ppm)]</th>
<th>24 h with addition of 10 h PCB 77&lt;sup&gt;d&lt;/sup&gt; [µM (ppm)]</th>
<th>10 h Concentration of PCB 77&lt;sup&gt;d&lt;/sup&gt; [µM (ppm)]</th>
<th>24 h Concentration of PCB 77&lt;sup&gt;d&lt;/sup&gt; [µM (ppm)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 77</td>
<td>—</td>
<td>5 (1.46)</td>
<td>0.7 (0.200)</td>
<td>0.2 (0.064)</td>
<td>0.7 (0.200)</td>
<td>0.7 (0.200)</td>
<td>0.2 (0.064)</td>
<td></td>
</tr>
<tr>
<td>PCB 37</td>
<td>—</td>
<td>—</td>
<td>0.2 (0.042)</td>
<td>0.2 (0.042)</td>
<td>0.1 (0.020)</td>
<td>0.1 (0.020)</td>
<td>0.1 (0.020)</td>
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<tr>
<td>PCB 35</td>
<td>—</td>
<td>—</td>
<td>0.2 (0.042)</td>
<td>0.2 (0.042)</td>
<td>0.1 (0.020)</td>
<td>0.1 (0.020)</td>
<td>0.1 (0.020)</td>
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<tr>
<td>PCB 15</td>
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<td>—</td>
<td>0.5 (0.110)</td>
<td>0.5 (0.110)</td>
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<tr>
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<td>—</td>
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<td>0.3 (0.076)</td>
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<tr>
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<tr>
<td>PCB 03</td>
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<td>—</td>
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<td>0.1 (0.028)</td>
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<tr>
<td>PCB 02</td>
<td>—</td>
<td>—</td>
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<td>0.1 (0.028)</td>
<td>0.1 (0.024)</td>
<td>0.1 (0.024)</td>
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<tr>
<td>Biphenyl</td>
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<td>—</td>
<td>1.9 (0.300)</td>
<td>1.9 (0.300)</td>
<td>3.1 (0.480)</td>
<td>3.1 (0.480)</td>
<td>3.1 (0.480)</td>
<td>5 (0.771)</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>5 (1.46)</td>
<td>5 (1.01)</td>
<td>4.3 (0.810)</td>
<td>5 (0.954)</td>
<td>4.8 (0.890)</td>
<td>5.5 (1.09)</td>
<td>5 (0.771)</td>
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</table>

<sup>a</sup> Treatment compositions representing different products at various points in the dechlorination process (see also Table 3.B.1)

<sup>b</sup> Treatment compositions representing dechlorination products at 10 and 24 h of dechlorination without the parent compound, PCB 77

<sup>c</sup> Treatment composition representing the dechlorination products of 24 h dechlorination with the increased concentration of PCB 77 present at 10 h of dechlorination

<sup>d</sup> Treatment composition representing the concentrations of PCB 77 present at 10 and 24 h of dechlorination

Concentrations listed represent final PCB concentrations in cell culture media.
**Figure 3.B.1.** Dechlorination of PCB 77 alters cellular oxidative stress. Cells grown on chamber slides were treated with DMSO (vehicle control) or dechlorination products with a total concentration of 5 µM total concentrations and were incubated for 4 h. Cells were then treated with N,N'-(1,2-dihydroxyethylene) bisacrylamide, (DHE, in red) for superoxide detection. Slides were fixed with DAPI (in blue) for nuclear staining, (A) fluorescent images were recorded at 40X and (B) DHE fluorescence was quantified and normalized to DAPI.
Figure 3.B.2. Dechlorination of PCB 77 attenuates NFκB activation. Cells were treated with vehicle control (DMSO), 0 h (PCB 77, 5 µM) 10 h, 24 h and 48 h dechlorination mixtures (5 µM), and incubated for 4 h. Both PCB 77 and dechlorination products (10 and 24 h product treatments) significantly increased DNA binding of NFκB over DMSO and biphenyl (48 h treatment). Results are given as mean±SEM. Different statistical marker letters represent significant differences among treatment groups (determined by P<0.05).
Figure 3.B.3. Dechlorination products alter CYP1A1 (A), MCP-1 (B) and VCAM-1 (C) mRNA expression. The presence of PCB 77 significantly up-regulated mRNA inflammatory markers in ECs; inflammation decreased with further dechlorination of the parent compound. Please see Table 3.B.2 for detailed treatment descriptions. The vehicle, DMSO (treatment 1), was applied at 0.1%v/v for all treatments. Treatments representing the dechlorination products at 0 h (treatment 2), 10 h (treatment 3), 24 h (treatment 5), and 48 h (treatment 8) are the same as those tested previously for NFκB, superoxide production and viability. Treatments 4 and 6 excluded PCB 77 while maintaining the representative concentrations of the dechlorination products found at 10 h and 24 h, respectively. In contrast, treatments 9 and 10 contained only PCB 77 at the concentrations found in the 10 h and 24 h mixtures, respectively. Treatment 7 consisted
of the 24 h dechlorination mixture with the level of PCB 77 found in the 10 h mixture. Different statistical marker letters represent significant differences among treatment groups (determined by $P_{\text{value}} \leq 0.05$). For example, in CYP1A1, treatment 2 (statistical marker “b”) is significantly different from treatment 1 (“a”) and treatment 4 (“c”).
Figure 3.B.4. Dechlorination mixtures alter CYP1A1 (A), MCP-1 (B) and VCAM-1 (C) protein expression. The presence of PCB 77 significantly up-regulated protein inflammatory markers in ECs; inflammation decreased with further dechlorination of the
parent compound. Treatments are the same as described in Figure 3.B.3 (please see Table 3.B.2 for detailed treatment descriptions). Statistical differences among treatment groups (determined by $P_{\text{value}} \leq 0.05$) are represented by marker letters, as stated previously.
Figure 3.B.5. PCB 77 increases ROS production and downstream cellular dysfunction. PCB 77 interacts with caveolae in the plasma membrane, is endocytosed into the cell, and interacts with the AhR and its chaperone ARNT (aryl hydrocarbon receptor nuclear translocater), leading to nuclear translocation, XRE (xenobiotic response element) binding and CYP1A1 up-regulation. CYP1A1 protein uncoupling occurs during PCB 77 metabolism and leads to an increase in cellular ROS. NFkB is activated in response to the change in cellular redox status and translocates to the nucleus where it acts as a transcription factor to up-regulate adhesion molecules and cytokines.
Chapter 4. Nutritional modulation of environmental pollutant in vivo toxicity

4.1 Synopsis

Superfund chemicals such as polychlorinated biphenyls pose a serious human health risk due to their environmental persistence and link to multiple diseases. Selective bioactive food components such as flavonoids have been shown to ameliorate PCB toxicity, but primarily in an in vitro setting. Here, we show that mice fed a green tea-enriched diet and subsequently exposed to environmentally relevant doses of coplanar PCB exhibit decreased overall oxidative stress primarily due to the up-regulation of a battery of antioxidant enzymes. C57BL/6 mice were fed a low fat diet supplemented with green tea extract (GTE) for 12 weeks and exposed to 5 µmol PCB 126/kg mouse weight (1.63 mg/kg-day) on weeks 10, 11 and 12 (total body burden: 4.9 mg/kg). F2-Isoprostane and its metabolites, established markers of in vivo oxidative stress, measured in plasma via HPLC-MS/MS exhibited five-fold decreased levels in mice supplemented with GTE and subsequently exposed to PCB compared to animals on a control diet exposed to PCB. Livers were collected and harvested for both mRNA and protein analyses, and it was determined that many genes transcriptionally controlled by AhR and Nrf2 proteins were up-regulated in PCB-exposed mice fed the green tea supplemented diet. An increased induction of genes such as SOD1, GSR, NQO1 and GST, key antioxidant enzymes, in these mice (green tea plus PCB) may explain the observed decrease in overall oxidative stress. A diet supplemented with green tea allows for an efficient antioxidant response in the presence of PCB 126 which supports the emerging paradigm that healthful nutrition may be able to bolster and buffer a physiological system against the toxicities of environmental pollutants.

4.2 Introduction

The contamination of soil and groundwater aquifers by toxic chlorinated organic compounds at Superfund sites, e.g., polychlorinated biphenyls (PCBs) and trichloroethylene (TCE), is a pervasive environmental problem with serious public health consequences. PCBs are persistent organic pollutants found in soil, air, and water, and a major source of human exposure to PCBs is dietary intake of contaminated foods. Because PCBs are lipid soluble, they readily accumulate in human tissues, thus increasing human health concerns. For example, the recent Aniston Community Health
Survey reported a significant correlation between PCB levels and risk of developing diabetes\textsuperscript{25}, and circulating levels of PCBs have also been associated with cardiovascular disease risk\textsuperscript{255}. Prenatal exposure to PCBs also may be associated with increased weight in children\textsuperscript{256}. The liver is particularly vulnerable to PCB-induced toxicity because it is the primary organ associated with detoxification, and there is strong evidence from NHANES data that exposure to PCBs is associated with liver disease in humans\textsuperscript{257}.

There is evidence from cell culture and animal models that nutrition can modulate the toxicity of environmental pollutants\textsuperscript{258} and thus affect vulnerability to environmental insults and compromised health. For example, PCBs can act as diet-dependent obesogens when administered with a high-fat diet, and thus worsen nonalcoholic fatty liver disease\textsuperscript{259}. We have previously shown that PCB exposure can modify lipid metabolism while dietary fat supplementation can ameliorate these negative effects\textsuperscript{260}. For example, we have shown that PCB exposure increases neutral lipid staining in LDL-R\textsuperscript{−/−} mice fed a corn oil-enriched diet (i.e., a diet rich in omega-6 fatty acids), which could indicate increased inflammation, while inflammation was decreased in mice fed an olive oil-enriched diet. Omega-3 fatty acids derived from fish oil are protective and reduce PCB-induced toxicity in endothelial cells\textsuperscript{48; 70}. Similarly, antioxidant nutrients such as dietary flavonoids can protect against endothelial cell damage mediated by these persistent organic pollutants\textsuperscript{60; 67}. This is important since coplanar PCBs (e.g., PCB 126) exert their toxicity primarily through activation of the aryl hydrocarbon receptor (AhR) and subsequent uncoupling of cytochrome P450 1A1 (CYP1A1), which can be a source of oxidative stress\textsuperscript{261; 262}.

Mammalian cells are constantly exposed to endogenous and exogenous sources of free radicals which tip the cellular balance towards an overall oxidative stress condition. To counteract the ubiquitous nature of reactive oxygen species (ROS), mammalian cells have evolved intricate and interrelated protein defenses that can work efficiently to limit the detrimental effects of these toxic molecules. PCBs have been shown to cause oxidative stress primarily through a CYP1A1 uncoupling mediated mechanism\textsuperscript{3}. Production of superoxide and related ROS triggers an up-regulation of a battery of antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase (GSR), glutathione transferases (GST), thioredoxins (Trx) and thioredoxin reductases (TrxR)\textsuperscript{263; 264}. These proteins work in concert to either catalyze the transformation of ROS to benign molecules such as water and molecular oxygen or
to reactivates enzymes, usually by catalytic reductions (e.g. TrxR reduces oxidized Trx to its active form). Such an interconnected system requires the crosstalk of multiple regulatory pathways including the aryl hydrocarbon receptor (AhR) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factors, which work together to detoxify xenobiotics and to up-regulate the antioxidant response (see Figure 4.1).

Upon activation via endogenous ligands, such as arachidonic acid metabolites, or xenobiotics, such as dioxin (TCDD) or coplanar PCB 126, the AhR translocates to the nucleus, binds consensus cis-acting sequences known as dioxin or xenobiotic response elements (DRE or XRE) and facilitates the up-regulation of multiple genes, especially those related to phase I detoxification (e.g. CYP1A1 and UDP-glucuronosyl S-transferases). Certain environmental toxicants with relatively long half-lives, such as TCDD and PCB 126, can promote sustained AhR activation resulting in chronic low levels of oxidative stress and inflammation. The Nrf2 pathway shares many target genes with AhR, but is generally regarded as a redox sensor, because its dissociation with inhibitory proteins (e.g. Keap1) and subsequent transactivation is promoted by ROS and electrophiles. Binding of Nrf2 to consensus antioxidant response elements (ARE) up-regulates a battery of protective genes including cytochrome P450s, GSTs and NAD(P)H dehydrogenase [quinone] 1 (NQO1). Nrf2 is a critical mediator of oxidative stress and xenobiotic toxicity as evidenced by multiple studies involving Nrf2 KO mice.

Importantly, bioactive nutrients such as tea catechins may work through both Nrf2- and AhR-mediated mechanisms to prevent toxicant-induced global inflammation. We have demonstrated previously that the tea catechin epigallocatechin-3-gallate (EGCG) can protect against vascular endothelial cell activation by coplanar PCBs, and that EGCG can inhibit expression of AhR-regulated genes and induce Nrf2-regulated antioxidant enzymes, thus providing protection against PCB-induced inflammatory responses in cultured endothelial cells. EGCG also can inhibit oxidative damage and attenuate carbon tetrachloride-induced hepatic fibrosis. Mechanisms responsible for EGCG-induced protection against environmental pollutants are not fully
understood. In the current study we provide evidence that green tea extract, composed primarily of EGCG (Supplementary Table 4.1), can decrease oxidative stress in livers of mice exposed to PCB 126 by a mechanism that, at least in part, is due to induction of antioxidant genes. Thus, diet supplementation with green tea may allow for an efficient antioxidant response to buffer against toxicities of environmental pollutants in humans and protect against PCB-induced liver damage\textsuperscript{257}.

4.3 Materials and Methods

4.3.1 Animals, diets, and dosing treatments

Forty C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 2 months of age and evenly assigned to the following experimental groups: control diet (10% kcal as fat) + vehicle, control+1% green tea extract (GTE) + vehicle, control + PCB 126, control+1% GTE + PCB 126. The control diet was purchased from Research Diets, Inc. (New Brunswick, NJ, catalog number D12450B). Sunphenon 30S-O organic green tea extract (lot number: 105131, containing 37.4% total polyphenols, 32.0% total catechins, and 5.3% caffeine) was obtained from Taiyo International Inc. (Minneapolis, MN) and incorporated into the control diet formulation, as described in Supplementary Table 4.1. Green tea extract treatment amounts per body weight coincide with approximately 4 cups of tea (~200 ml/cup) per day in humans\textsuperscript{270}. Mice were fed the control and GTE-supplemented diets for 12 weeks and were gavaged with PCB 126 (5 µmol/kg mouse) or vehicle (stripped safflower oil; Acros Chemical Company, Pittsburgh, PA) in weeks 10, 11, and 12. The PCB 126 gavage concentration was chosen based on observations in preliminary studies where gavage of 5 µmol PCB 126/kg mouse weight (1.63 mg/kg-day, total body burden: 4.9 mg/kg) showed pro-inflammatory responses in C57/BL6 mice but not wasting syndrome.

4.3.2 Blood and tissue harvesting

In this study, we examined the role that green tea extract catechins play in altering oxidative stress and inflammation following insult with environmental pollutants (i.e., PCB 126). 24 h after week 12 treatment, mice were euthanized with CO\textsubscript{2} and quickly exsanguinated. Ethylenediaminetetraacetic acid (EDTA) was added to collected blood samples, briefly mixed, and centrifuged at 5000 g for 5 min at 4 °C to separate blood plasma. Plasma samples were frozen in liquid nitrogen and stored at -80 °C until processing. Livers were harvested, weighed, divided in half, and frozen in liquid nitrogen.
for protein studies or stored in RNAlater solution (Life Technologies, Grand Island, NY) at 4 °C for 24 h then -80 °C prior to mRNA analysis.

4.3.3 Plasma PCB and isoprostane analysis

PCB 126 and its metabolites were extracted from plasma samples to determine systemic PCB and metabolite concentrations and correlate these findings to potential PCB-induced oxidative stress as well as the role of green tea extract in mitigating these effects. PCB 126 and its hydroxy metabolites were isolated from plasma samples (plus 10 μM 13C12-labeled PCB 126 internal standard (IS), Cambridge Isotope Laboratories, Tewksbury, MA) through extraction with acetonitrile and subsequent sonication and centrifugation at 15,000 rpm for 5 min to pellet plasma debris. Supernatants were dried under N₂ and reconstituted in 99:1 methanol:dl H₂O solvent mixture with 0.5% formic acid and 0.1% 5 M ammonium formate.

Measurement of F₂-Isoprostanes (F₂-IsoPs) provides one of the most reliable assessment methods for oxidative stress in vivo. For F₂-IsoP analysis, plasma samples were added to 5:1 ethyl acetate: methanol + 0.5% acetic acid (v/v) + 10 μM 8-iso-PGF2α-D4 (internal standard, Cayman Chemical, Ann Arbor, MI), vortexed briefly, and centrifuged to pellet plasma debris. Supernatants were transferred and dried under N₂ prior to reconstitution in methanol and addition of acetic acid for subsequent solid phase extraction (SPE).

Reconstituted F₂-IsoP samples were loaded onto pre-conditioned Supel-Select HLB SPE columns (Sigma-Aldrich, St. Louis, MO) and washed with 0.5% acetic acid followed by washing with 0.5% acetic acid containing 20% methanol. Columns were eluted with methanol, eluent was evaporated to dryness with N₂, and samples were reconstituted with 50:50 methanol:dl H₂O.

Plasma PCB 126 and a hydroxy metabolite as well as extracted plasma F₂-IsoPs were analyzed using a Shimadzu ultra fast liquid chromatography (UFLC) system coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. MRM transitions monitored: 325.9/256.1, 325.9/254.1, and 325.9/184 for PCB 126; 338/268.1, 338/196.1, and 338/265.7 for 13C12 PCB126. In the MRM ion transition, the precursor ion represents the M⁺· and the product ion represents either [M-Cl]⁺ or [M-2Cl]⁺. MRM transitions monitored with regard to hydroxy PCB metabolites: 340.8/340.9 for hydroxy PCB126
and 386.8/340.9 for dihydroxy PCB126. The precursor ion of the ion transition is a formic acid adduct: [M+FA-H]⁻ and product ion is [M-H]-. F₂-IsoP were analyzed by integrating peak area (area under the curve, AUC) with regard to known internal standard concentrations (AUC/IS). All values were subsequently normalized for sample volume and compared to ion transitions of internal standard (13C12 PCB 126) with known concentration to determine PCB parent and metabolite concentrations (pmol/µL plasma).

4.3.4 RNA isolation and polymerase chain reaction (PCR) amplification

Liver samples used to analyze oxidative stress and inflammatory mRNA markers were homogenized and mRNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. mRNA concentrations were then determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, WI). mRNA levels were determined by quantitative real-time PCR using a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems) as compared to constitutively expressed β-actin (forward primer: 5’-TGTCACCCCTCCAGAGATGT-3’; reverse primer: 5’-GCTCAGTACAGTCCGCTAGAA-3’) using the relative quantification method (ΔΔCt). Primer sequences (see Table 4.1) for SYBR Green reactions were designed using the Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

4.3.5 Immunoblotting

Liver samples used for protein analysis were homogenized in extraction RIPA buffer containing protease inhibitors (Pierce, Rockford, IL). Lysed tissue was centrifuged at 10,000 g for 30 min at 4 °C followed by Bradford protein assay (Pierce). Protein samples were separated using 10% SDS-PAGE and subsequently were transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk buffer and incubated overnight at 4 °C with the following primary antibodies: β-actin (product #A2066, ~42 kD, Sigma, St. Louis, MO), GAPDH (product #sc-20357, ~37 kD, Santa Cruz Biotechnology, Dallas, TX), GSR (product #ab16801, ~58 kD, Abcam, Cambridge, MA), and NQO1 (product #ab34173, ~31 kD, Abcam). After washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and visualized using ECL detection reagents (Thermo, Waltham, MA).
Liver samples used for nuclear translocation assays were prepared and cytoplasmic and nuclear proteins were extracted according to manufacturer protocol (NE-PER® Nuclear and Cytoplasmic Extraction Method, Thermo). Translocation samples subsequently were processed for Western blotting as described above, and probed with the following primary antibodies: lamin (product #sc-7292, ~69 kD, Santa Cruz Biotechnology) and Nrf2 (product #sc-722, ~59 kD, Santa Cruz Biotechnology).

4.3.6 Data analyses

Data were analyzed using SigmaStat software (Systat Software, Point Richmond, CA). Comparisons between treatments were made by one-way or two-way ANOVA with post-hoc comparisons of the means. Overall, few statistical differences were exhibited between vehicle control diet groups, thus in most cases Student's t-test was used to analyze differences between PCB treatment groups. qRT-PCR mRNA analysis (n=8-10), Western blot protein analysis (n=8), and nuclear translocation analysis (n=4) represent three experimental replicates. A probability value of p≤0.05 was considered statistically significant.

4.4 Results

4.4.1 Systemic toxicity associated with PCBs

PCB 126 levels in mouse plasma were examined as a measure of systemic PCB body burden and to determine the possible effect of green tea extract (GTE) diet supplementation on PCB metabolism/excretion. As seen in Figure 4.2, plasma PCB 126 was found almost completely as its hydroxylated metabolite, OH-PCB 126, with concentrations of approx. 0.04 pmol PCB 126/µL plasma versus approx. 30 pmol OH-PCB 126/µL, respectively. GTE diet supplementation did not modulate PCB metabolism or plasma concentrations 24 h following PCB exposure, indicating that it plays a minimal role in pollutant clearance from the body. Additionally, Supplementary Figure 4.1 shows that while PCB treatment led to a significant increase in liver/body weight ratio (hepatosomatic index, p<0.001), in both control and GTE-supplemented diets, GTE supplementation did not significantly mitigate this PCB-induced increase.
4.4.2 \textit{F}_2\text{-isoprostane levels are significantly reduced in green tea extract-supplemented, PCB-exposed mice}

Analysis of \textit{F}_2\text{-isoprostanes (F}_2\text{IsoPs), prostaglandin-like eicosanoids formed during fatty acid peroxidation, has emerged as the most reliable method for assessing in vivo oxidative stress}^{271}. Plasma samples from mice fed control and GTE-supplemented diets and subsequently treated with vehicle or PCB 126 (n=8-10) were analyzed to determine GTE’s role in modulating environmental toxicant-induced oxidative stress. Plasma \textit{F}_2\text{-IsoP (including PGF2}_\alpha\text{, 8-isopGF2}_\alpha\text{, iPFP2}_\alpha\text{-III, 8-epiPGF2}_\alpha\text{, 8-isoprostane, and 15-F2t isoprostanes) and F}_2\text{IsoP metabolite (13,14-dihydro-15-ketoPGF2}_\alpha\text{) concentrations were determined. As seen in Figure 4.3, GTE diet supplementation led to drastically decreased F}_2\text{-IsoP levels (approximately a five-fold reduction, p<0.05) in mice treated with PCB 126, indicating that GTE acts as a strong antioxidant to modulate against environmental toxicant insult. Additionally, GTE drastically decreased PCB-induced F}_2\text{-IsoP metabolite production (greater than a five-fold reduction, p<0.05); F}_2\text{-IsoP metabolite analysis is developing as an even more sensitive measure of in vivo oxidative stress because the metabolites do not undergo autoxidation and artificial production as has been seen with parent F}_2\text{-IsoP}^{272}. Interestingly, GTE supplementation led to no significant modulation of F}_2\text{-IsoP parent or metabolite levels under control situations, indicating that antioxidant modulation occurs primarily when a system is under a secondary stressor.}

4.4.3 \textit{Green tea extract increases antioxidant gene expression}

Antioxidant enzyme levels were measured in mouse liver to further develop the role of GTE diet supplementation in modulating environmental insults in vivo. Table 4.2 highlights antioxidant mRNA markers tested and overall results. qRT-PCR analysis (n=8-10) shows a significant up-regulation in catalase, glutathione peroxidase (Gpx3), glutaredoxin (Grx2), glutathione reductase (GSR), glutathione S-transferases (GSTa1, GSTa4, GSTm1, GSTm2, and GSTm3), NAD(P)H dehydrogenase [quinone] 1 (NQO1), superoxide dismutase 1 (SOD1), thioredoxin 2 (Trx2), and thioredoxin reductase 1 (TrxR1) during the concomitant treatment of PCB 126 and GTE diet supplementation. As before, in most cases GTE supplementation did not significantly modulate antioxidant response in the absence of PCBs. NQO1 and GSTm3, enzymes associated with detoxification, exhibited significantly increased mRNA levels above vehicle control diet levels in the presence of PCB 126, while GTE diet supplementation drastically induced
antioxidant mRNA expression following PCB insult. mRNA levels of SOD1, critical for modulating harmful superoxide radicals produced during toxicant insult, were significantly decreased following PCB gavage, while GTE supplementation returned mRNA expression to vehicle control diet levels. While PCB administration did not modulate GSR (an important cellular antioxidant) mRNA levels in mice fed vehicle control diets, GTE diet supplementation led to a significantly increased antioxidant response (see Figure 4.4, p<0.01). Additional data shown in Supplementary Figure 4.2 is also consistent with these trends observed in response to GTE supplementation. For example, thioredoxin 2 (Trx2, an important redox protein) mRNA levels are significantly up-regulated in the concomitant presence of GTE and PCB 126 although GTE does not induce increased antioxidant activity without the addition of secondary external insult.

4.4.4 Green tea extract increases NQO1 and GSR antioxidant protein response against PCB 126

Antioxidant marker protein analysis was performed in order to better understand the role that GTE diet supplementation plays in increasing the body’s defensive mechanisms against toxicant insult. Proteins of interest were normalized to multiple housekeeping genes, β-actin and GAPDH. In PCB 126-treated mouse liver samples, NQO1 protein was significantly up-regulated when fed a GTE supplemented diet, as shown in Figure 4.5 when quantified against GAPDH (p<0.01). The associated representative blot continues trends seen in mRNA with a large increase in antioxidant protein activity in GTE supplemented mice exposed to PCB insult. GSR protein expression was also statistically increased in response to diet supplementation and continues the trend seen in mRNA analysis in which neither GTE supplementation nor PCB treatment led to modulation of antioxidant response while their concomitant treatment led to significant up-regulation (p≤0.05).

4.4.5 Green tea extract drives Nrf2 nuclear translocation in the presence of PCB 126

Nuclear translocation assays are commonly used techniques that serve as a representation of cellular transcriptional activation. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) nuclear translocation was analyzed in comparison to lamin, a nuclear fraction housekeeping gene; liver samples from GTE-supplemented mice exposed to PCB 126 showed a trend toward increased nuclear abundance of Nrf2 (p=0.1, n=4). As
in antioxidant mRNA and protein studies, GTE without a concomitant insult from PCB did not modulate Nrf2 activity and translocation. The Nrf2 antioxidant pathway plays a pivotal role in modulating oxidative stress, and therefore its up-regulation by GTE diet supplementation, as seen in Figure 4.6, is an important indicator of GTE’s ability to increase the body’s responsiveness toward environmental stressors.

4.4.6 Green tea extract modulates inflammatory and xenobiotic-related markers in the presence of PCB 126

Cytochrome P450 (e.g., CYP1A1 and CYP1B1), a family of enzymes controlled by the aryl hydrocarbon receptor (AhR) and Nrf2 proteins that are vital for the metabolism of xenobiotic substances, including toxicants, was analyzed from liver samples to determine potential modulation of its activity in response to GTE diet supplementation. Substantial CYP up-regulation was seen in the presence of PCB insult, as has been shown previously, while concurrent GTE supplementation causes a significant up-regulation in CYP1B1 mRNA analysis (as seen in Figure 4.7). In mice fed GTE-supplemented diets and exposed to PCB, major indicators of inflammation including monocyte chemoattractant protein-1 (MCP-1, also referred to as chemokine (C-C motif) ligand 2, CCL2) and CCL3 (also referred to as macrophage inflammatory protein-1α, MIP-1α) were significantly decreased back to vehicle control levels. Interestingly, GTE supplementation alone led to significant increases in inflammatory marker mRNA levels, although these levels returned to vehicle control levels with concomitant treatment (p<0.05).

4.4.7 The AhR is implicated as a control mechanism both in PCB 126 toxicity and antioxidant response

As seen in Figure 4.8, GTE diet supplementation led to significant up-regulation of AhR mRNA levels in liver in the presence of PCB 126, a dioxin-like AhR ligand. PCB 126 insult did not induce AhR mRNA in vehicle control, vehicle + GTE control, and PCB control settings, but, interestingly, concomitant treatment led to a two-fold up-regulation, similarly to that seen in mRNA and protein antioxidant and inflammatory markers, thus allowing for increased in vivo toxicant clearance (p<0.01). Nrf2 mRNA levels were also significantly increased during PCB 126 insult, although GTE supplementation did not cause significant modulation of PCB toxicity. AhR and Nrf2 signaling pathways control both xenobiotic responses and inflammatory cascades, therefore their modulation by
GTE diet supplementation implicates further GTE’s role in strengthening antioxidant response toward insult by environmental pollutants.

4.5 Discussion

Healthy nutrition can positively influence, or lessen, the human health risks associated with exposure to mixtures of environmental chemicals\(^{273}\). The liver is one tissue particularly vulnerable to environmental pollutants, and especially PCB-induced toxicity\(^{257}\). One type of liver disease that affects more than 20% of Americans is nonalcoholic fatty liver disease, which can lead to nonalcoholic steatohepatitis\(^ {274}\). Industrial toxicants also have been linked to secondary insults that can lead to steatohepatitis\(^ {274}\). Our laboratory and others have shown that PCB exposure increases liver/body weight ratio (hepatosomatic index, HSI; see Supplementary Figure 4.1), which is generally accepted as indicative of an adverse hepatic response to chemical exposure\(^ {275}\). Mechanisms defining the involvement of environmental pollutants in the pathology of liver diseases may include a compromised redox status or toxicant-induced increase in oxidative stress.

Lifestyle modifications that include healthful nutrition have been suggested as a powerful means of reducing the vulnerability to environmental insults\(^ {273}\) and in reducing the risk to environmental toxicant-induced liver disease\(^ {274}\). For example, green tea extract has been shown to protect against hepatic steatosis in obese mice by reducing oxidative stress and enhancing hepatic antioxidant defenses\(^ {276}; 277\). In the present study we observed significant protection against PCB 126-induced oxidative stress by dietary supplementation with green tea extract. In fact, mice supplemented with green tea extract and subsequently exposed to PCB displayed a decrease in overall F\(_2\)-isoprostane and metabolite levels compared to animals on a control diet exposed to PCB. We did not see a large increase in oxidative stress as evidenced by an increase in F\(_2\)-isoprostane levels in mice fed a control diet and subsequently gavaged with PCB. This may have been due to the fact that the amount of administered PCB 126 reflects more relevant environmental PCB exposure concentrations in humans than previous studies using high concentrations of PCB congeners (e.g., 150 µmol/kg in previous studies versus 5 µmol/kg used herein)\(^ {278}; 279\). We hypothesize that levels of PCBs encountered by humans today initiate low levels of chronic oxidative stress and inflammation that, together with multiple other factors including poor diet and exposures
to other environmental stressors, leads to augmented or exacerbated human disease. The protective properties of green tea have been studied extensively, and recent human studies suggest that consumption of green tea may protect against cardiovascular disease and some forms of cancer, have anti-hypertensive and anti-obesity effects, and contribute to antibacterial and antiviral activity.

Certain environmental pollutants, and in particular ligands for the AhR, induce oxidative stress, in part via induction and uncoupling of cytochrome P450 enzymes. Hepatic changes in lipid composition, altered membrane structure and membrane functions are well-described phenomena of PCB-induced liver damage. The protective properties of green tea extracts against PCB-induced pathologies, and in particular liver damage, may be numerous. For example, green tea may normalize the formation of lipid peroxide products induced by exposure to toxicants to prevent hepatic fibrosis, or green tea may favorably regulate intestinal tight junction proteins or overall intestinal barrier function. It has been proposed that green tea can inhibit the intestinal absorption of lipids and highly lipophilic organic compounds. Induction of antioxidant enzymes by green tea also may contribute to its tissue protective properties. Our current study supports this concept, and we observed an increased induction of antioxidant genes such as SOD1, GSR, NQO1 and GST in mice that were fed a green tea-supplemented diet and subsequently challenged with PCB, compared to animals exposed to the control diet plus PCB. This may explain in part the observed decrease in overall oxidative stress due to green tea supplementation. Of particular interest is our observed induction of NQO1, a Nrf2 target gene, which suggests that green tea protects in part by modulation of the Nrf2/ARE pathway. In fact, it has been shown that food polyphenols, including the green tea polyphenol EGCG, the primary component of GTE tested herein, can modulate Nrf2-mediated antioxidant and detoxifying enzyme induction. Our own work with vascular endothelial cells further suggests that multiple pathways including lipid raft caveolae and the antioxidant defense controller Nrf2 play a role in nutritional modulation of PCB-induced vascular toxicity and that cross-talk between caveolae-related proteins and cellular Nrf2 may be required for optimal cytoprotection by green tea catechins and other diet-derived polyphenols.

Many groups, including ours, have shown that green tea catechins such as EGCG can up-regulate basal levels of antioxidant enzymes in vitro. Interestingly, our overall results show that green tea extract supplemented in the diet acts as an
antioxidant only in the presence of a secondary stressor, in this case, the pro-
inflammatory coplanar PCB 126. The inconsistencies between in vitro and in vivo studies
may be explained by the relatively high doses of tea catechins usually employed in cell
culture or the fact that most tea catechins are quickly biotransformed in vivo to
metabolites that exhibit differential physiological effects\textsuperscript{286}. There are many other
examples of instances where supplementation with GTE or specific catechins is
protective in in vivo models of inflammation and oxidative stress. For many of our
investigated antioxidant enzymes we saw decreased expression in the presence of PCB
when fed a control diet, but levels were up-regulated, many returning to control vehicle
levels in PCB groups fed a GTE-rich diet (e.g., SOD1 in Figure 4.4). These observations
are in line with other groups who investigated GTE effects on other stressors including
ethanol toxicity and bacterial infection\textsuperscript{290; 291}.

Our past work in cell culture points to the antioxidant controller Nrf2 as a major
player in nutritional modulation of PCB toxicity. Many nutrients other than green tea
catechins, including resveratrol, found in the skins of grapes, and sulforaphane, which is
found in broccoli, have been shown to activate Nrf2\textsuperscript{74; 87; 88; 290; 291}. Nrf2 can become
transcriptionally active through multiple mechanisms including direct phosphorylation by
PKC delta and loss of contact between Nrf2 and inhibitory kelch-like ECH-associated
protein 1 (Keap1)\textsuperscript{89}. Upon activation, Nrf2 is able to evade ubiquitination, enter the
nucleus, and bind \textit{cis}-acting antioxidant response elements in target genes such as
NQO1\textsuperscript{90}. Nrf2 activation leads to decreased overall oxidative stress and inflammation,
which is a hallmark of PCB toxicity\textsuperscript{86}. In this work we observed a relatively significant
trend toward increased NRF2 translocation to the nucleus in animals supplemented with
GTE and subsequently exposed to PCB (Figure 4.6). More interestingly and novel, we
also saw a drastic increase in AhR mRNA expression in this same treatment group
(Figure 4.8). This up-regulation was mirrored in increases in both CYP1A1 and CYP1B1
mRNA levels in mice fed a GTE rich diet and subsequently exposed to PCB. An
increase in AhR may help to detoxify the acute exposure to PCB by increasing
metabolism-assisted excretion. Although a consistent, steady up-regulation of AhR may
create a negatively imbalanced redox situation, the GTE’s ability to up-regulate AhR only
in the presence of a toxicant may in some cases be a protective and positive
mechanism. Other groups have shown that different catechins within GTE display either
antagonistic or agonist activities against CYP1A1\textsuperscript{292}, but to our knowledge no group has
reported the mRNA up-regulation as seen in Figure 4.7. In our analysis of PCB
concentrations in plasma we observed a very slight trend towards decreased levels of parent PCB 126 in the plasma of mice supplemented with GTE (Figure 4.2). Although plasma levels may be a good overall picture of body-burden of PCBs, in the future, collecting urine and feces may paint a clearer picture of GTE’s involvement with detoxification and excretion. Also, we may have been able to see a more significant decrease in PCB levels and or a modulation of PCB hydroxy metabolite in mice supplemented with GTE if we sacrificed the mice more than 24 hours after the final PCB dose.

PCBs can induce vascular inflammation by up-regulating pro-inflammatory mediators such as MCP-1 and CCL3. We hypothesized that GTE would down-regulate basal levels of these inflammatory markers in vehicle treated mice as well as decrease PCB-mediated up-regulation in PCB 126 treated mice. Interestingly however, for both of our inflammatory markers we saw a significant increase in mRNA levels in vehicle treated mice supplemented with GTE (Figure 4.7). This observation would suggest that the dose of GTE used in this study may not be optimal, and perhaps toxic to some degree, in basal levels of oxidative stress and inflammation. Other groups have shown GTE toxicities at certain doses in vivo, and interestingly, data illustrating protection seems to be more conclusive in animal models of oxidative stress and inflammation. Importantly, for our study, both MCP-1 and CCL3 mRNA levels return to vehicle treated control diet levels in mice fed GTE and subsequently exposed to PCB. This may point to GTE as exhibiting possible hormetic activity by inducing a slight response by the organism that ultimately primes the protective antioxidant system for a future stressor, i.e., Superfund pollutant exposure. Understanding hormesis and the role that nutrients can play is an extremely interesting scientific discipline and demands much more future investigation.

In summary, our current study supports our in vitro data that green tea catechins can protect against PCB 126-induced cytotoxicity by reducing oxidative stress. Our current in vivo data contributes to the overall hypothesis that nutrition can modulate environmental insults. More studies are needed to further understand detailed mechanisms of protective benefits to consume diets high in protective and healthful nutrients such as plant-derived polyphenols and other bioactive compounds. (The data presented in Chapter 4 were published.)
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
<th>Fragment size</th>
</tr>
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<tbody>
<tr>
<td><strong>Inflammatory and xenobiotic-related markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AhR</td>
<td>GACCAAACACACAAAGCTAGAATTCACACC</td>
<td>CAAGAAGCGGAAAACACTGCATGCT</td>
<td>200 bp</td>
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<tr>
<td>CCL3</td>
<td>CACCCCTGTGACCACTCTGCTCAA</td>
<td>TGGCGCTGAGAAGACTCTGCT</td>
<td>100 bp</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>TGGAGCTCTCCCGAGATCTCTCTC</td>
<td>CATACATGGAGAGCACTGGTAAAGCT</td>
<td>100 bp</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>TGATCTCGAGGAGAACGGTCTCTCTC</td>
<td>TCTACTTCTGGAGATCATCTGCT</td>
<td>104 bp</td>
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<tr>
<td>MCP-1</td>
<td>GCAATCTGAGGAGAACGGTCTCTCTC</td>
<td>CCTACTTCTGGAGATCATCTGCT</td>
<td>63 bp</td>
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<tr>
<td>Nrf2</td>
<td>GAGTCGCTTGCCTGTGATCTTCTCTC</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
<td>100 bp</td>
</tr>
<tr>
<td><strong>Antioxidant markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>CAGAGAGCGGATTCTCTGAGAAGAATCTC</td>
<td>CTTGACTCTTGAGAGATCATCTGCT</td>
<td>100 bp</td>
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<tr>
<td>Gpx3</td>
<td>TGAGCTCTGAGGAGAACGGTCTCTCTC</td>
<td>CAGTTTTCTTGTGAGATCATCTGCT</td>
<td>125 bp</td>
</tr>
<tr>
<td>Grx2</td>
<td>CATCAGCTTACTCTTCTCACCATGCT</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
<td>123 bp</td>
</tr>
<tr>
<td>GSR</td>
<td>TCGGAATTCATGACAGATCTCTCTC</td>
<td>GAGCCACGGAACATCATACAT</td>
<td>100 bp</td>
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<tr>
<td>GSTa1</td>
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<td>CTTGACTCTTGAGGAGACCATGCAAGAA</td>
<td>159 bp</td>
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<tr>
<td>GSTa4</td>
<td>TACCTCGAGGAGAACGGTCTCTCTC</td>
<td>CAGTTTTCTTGTGAGATCATCTGCT</td>
<td>109 bp</td>
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<tr>
<td>GSTm1</td>
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<td>AGTCAGGCTTGTGAGGAGACCAT</td>
<td>349 bp</td>
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<tr>
<td>GSTm2</td>
<td>CATCAGCAGGCTCTGAGGAGAACGGTCTCTCTC</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
<td>118 bp</td>
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<td>GSTm3</td>
<td>CCCACAGCTGTGAGGAGAACGGTCTCTCTC</td>
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<tr>
<td>NQO1</td>
<td>GCAATCGGATCGCTGAGGAGAACGGTCTCTCTC</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
<td>100 bp</td>
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<tr>
<td>SOD1</td>
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<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
<td>100 bp</td>
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<tr>
<td>Trx2</td>
<td>GCCTACCGGATCTGAGGAGAACGGTCTCTCTC</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
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<tr>
<td>TrxR1</td>
<td>GCCTACCGGATCTGAGGAGAACGGTCTCTCTC</td>
<td>TCAATGGCTGAGGAGACCATGCAAGAA</td>
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Table 4.2
The effect of green tea extract (GTE) diet supplementation on PCB 126-induced mRNA inflammatory, xenobiotic-related and antioxidant markers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Control diet</th>
<th>Control + 1% GTE</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>Inflammatory and xenobiotic-related markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AhR</td>
<td>0.771±0.096</td>
<td>1.506±0.131</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CCL3</td>
<td>2.051±0.224</td>
<td>0.945±0.116</td>
<td>&lt;0.001</td>
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<td>CYP1A1</td>
<td>916.208±136.510</td>
<td>1169.338±78.900</td>
<td>N.S.</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>87.504±7.694</td>
<td>146.998±12.329</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.909±0.478</td>
<td>0.745±0.235</td>
<td>0.012</td>
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<tr>
<td>Nrf2</td>
<td>3.088±0.307</td>
<td>3.212±0.234</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Antioxidant markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>0.495±0.057</td>
<td>0.817±0.071</td>
<td>0.003</td>
</tr>
<tr>
<td>Gpx2</td>
<td>0.339±0.161</td>
<td>0.682±0.098</td>
<td>0.08</td>
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<tr>
<td>Gpx3</td>
<td>0.925±0.167</td>
<td>1.411±0.159</td>
<td>0.004</td>
</tr>
<tr>
<td>Grx2</td>
<td>0.213±0.015</td>
<td>0.486±0.078</td>
<td>0.003</td>
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<tr>
<td>GSR</td>
<td>0.702±0.074</td>
<td>1.245±0.097</td>
<td>0.001</td>
</tr>
<tr>
<td>GSTa1</td>
<td>14.771±2.911</td>
<td>22.955±3.975</td>
<td>0.034</td>
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<td>GSTa4</td>
<td>0.896±0.117</td>
<td>2.222±0.245</td>
<td>&lt;0.001</td>
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<tr>
<td>GSTm1</td>
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<td>4.024±0.301</td>
<td>0.006</td>
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<tr>
<td>GSTm2</td>
<td>0.868±0.097</td>
<td>1.366±0.114</td>
<td>0.004</td>
</tr>
<tr>
<td>GSTm3</td>
<td>4.469±0.664</td>
<td>18.596±1.819</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NQO1</td>
<td>1.980±0.051</td>
<td>6.138±0.031</td>
<td>0.006</td>
</tr>
<tr>
<td>SOD1</td>
<td>0.311±0.041</td>
<td>0.684±0.063</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trx2</td>
<td>0.731±0.050</td>
<td>1.556±0.152</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TrxR1</td>
<td>1.522±0.143</td>
<td>2.673±0.276</td>
<td>0.002</td>
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</tbody>
</table>
Figure 4.1. Proposed signaling pathway for PCB detoxification in vivo. PCB 126, an AHR ligand and activator of NRF2, causes CYP1A1 up-regulation, which leads to superoxide production. Green tea extract (GTE) diet supplementation effectively up-regulates redox-related enzymes in the presence of PCB 126 which allows for a more efficient antioxidant response to environmental insult.
**Figure 4.2.** The effect of green tea extract (GTE) diet supplementation on systemic PCB 126 concentration and metabolism. PCB 126 and its hydroxy metabolites were measured in mouse plasma by UFLC/MS MS and normalized to sample volume and internal standard recovery. PCB 126 is heavily metabolized *in vivo*, as seen by very low levels of parent PCB 126 remaining in plasma samples while its hydroxylated metabolites predominate. GTE supplementation did not significantly modulate systemic PCB or metabolite concentrations. Data are presented as mean±S.E.M (n=5).
Figure 4.3. PCB 126-induced oxidative stress is modulated by green tea extract (GTE) diet supplementation. Plasma $F_2$-isoprostane (A) and metabolite (B) levels were measured by HPLC/MS MS to assess in vivo oxidative stress induced by PCB 126 that is potentially mitigated by GTE supplementation. Relative levels of combined F-2 IsoPs, including PGF2α, 8-iso-PGF2α, iPF2α-III, 8-epiPGF2α, 8-isoprostane, and 15-F2t isoprostanes, were determined by averaging the AUC integration values from retention times of 8 and 11.3 minutes (Q1 = 353.144, Q2 = 193.1). Additionally, the level of 13,14-dihydro-15-ketoPGF2α, an F-2 IsoP metabolite, was determined by integrating its peak at 11.3 minutes (Q1 = 355.2, Q2 = 311.4). Data are presented as mean±S.E.M. (n=8-10). GTE supplementation significantly decreased oxidative stress induced by PCB 126 treatment (*p<0.05).
Figure 4.4. Relative mRNA levels of representative antioxidant enzyme markers, NQO1 (A), GSTm3 (B), SOD1 (C) and GSR (D) in mouse liver samples. Overall GTE supplementation did not significantly increase antioxidant mRNA levels in control diets, but, in the presence of environmental perturbation (i.e. PCB 126 gavage), significantly higher antioxidant levels were seen in mouse liver above non-supplemented diet. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M (*p<0.01, n=8-10). See Table 4.2 and Supplementary Figure 4.1 for more information concerning all antioxidant markers tested.
Figure 4.5. GTE supplementation leads to increased antioxidant protein expression in the presence of PCB 126. Protein expression of NQO1 (A) and GSR (B) in mouse liver samples was assessed by Western blot analysis. Protein samples were separated through gel electrophoresis and probed with NQO1 and GSR primary antioxidant-related antibodies. Statistically significant increases in antioxidant protein activity were seen in PCB 126-treated mice that were fed a GTE-supplemented diet. In addition to visualized Western blot comparison to β-actin housekeeping gene, samples were compared to GAPDH housekeeping gene for densitometry quantification to further substantiate findings. GTE supplemented mice exposed to PCB showed a significant increase in protein expression, indicating a strengthened antioxidant response due to GTE supplementation (*p<0.01, **p≤0.05, N=8).
Figure 4.6. Nuclear translocation of Nrf2 in mouse liver samples. Mice fed a 1% GTE-supplemented diet and subsequently exposed to PCB 126 displayed increased Nrf2 activation, as evidenced by increased Nrf2 translocation to the nucleus, compared to mice fed 10% fat control diet and exposed to PCB. Lamin was used as a nuclear fraction housekeeping gene for densitometry quantifications. GTE supplemented mice exposed to PCB showed a trend toward increased nuclear abundance of Nrf2 (*p=0.1, n=4).
Figure 4.7. Relative mRNA levels of inflammatory and xenobiotic-related markers, Cyp1A1 (A), Cyp1B1 (B), MCP-1 (C) and CCL3 (D) in mouse liver samples. GTE supplementation led to increased cytochrome P450 (CYP1A1 and CYP1B1) mRNA expression in the presence of both GTE and environmental toxicant (i.e., PCB 126), indicating increased activity for toxicant degradation and/or excretion. MCP-1 and CCL3 inflammatory markers were statistically increased in GTE-supplemented mice liver samples but toxicant-induced inflammatory markers returned to control levels due to GTE supplementation. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M. (*p<0.01, **p<0.05, n=8-10).
Figure 4.8. mRNA expression for AhR (A) and Nrf2 (B) genes in mouse liver samples. GTE diet supplementation leads to significant up-regulation of AhR mRNA levels in the presence of PCB 126, thus increasing *in vivo* toxicant clearance. Nrf2 mRNA levels are significantly increased during PCB 126 insult, although GTE supplementation does not cause statistically significant modulation of PCB toxicity. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M (n=8-10). GTE supplementation significantly increased AhR in the presence of PCB 126 treatment (*p<0.01).
Chapter 5. Conclusions

5.1 Overall outcomes

In conclusion, we have demonstrated that aluminum oxide nanoparticles can be effectively stabilized under biologically relevant conditions by optimizing charge stabilization based on buffer characteristics and pH, and by tailoring ultrasonic sheer to initially disperse aggregated particles as single nanoparticles or small agglomerates. Citrate buffer, pH 6.0, with high sheer probe ultrasonication produces alumina nanoparticle suspensions in the nanosize regime (below 100 nm) that are stable for extended periods of time in buffer or when added to relevant biological media for eventual in vitro toxicity studies.

Nanotoxicology studies were performed to study potential concerns associated with increased nanoparticle usage in industrial and biomedical settings. Titanium dioxide nanoparticles, another highly relevant metal oxide particle system used heavily in industrial and commercial applications, were tested to determine overall in vitro effects in vascular endothelial cells and to further understand the cellular signaling pathways involved in TiO$_2$-linked oxidative stress and inflammation. TiO$_2$ NP exposure was shown to increase cellular oxidative stress and DNA binding of NFκB, significantly increase induction of mRNA and protein levels of VCAM-1 and MCP-1 inflammatory markers, and increase phosphorylation of key signaling pathways linked to NFκB activation and subsequent inflammation. These data indicate that TiO$_2$ NPs can induce endothelial inflammatory responses via redox-sensitive cellular signaling pathways.

The toxicity of PCB 77, a coplanar persistent environmental pollutant that exhibits dioxin-like toxicity, was studied at various stages of iron/palladium nanoparticle-induced dechlorination to determine the effectiveness of PCB degradation for decreasing associated public health risk. Although complete PCB 77 degradation to biphenyl was shown to eliminate PCB-induced endothelial dysfunction, degradation product mixtures showed significant in vitro toxicity in vascular endothelial cell systems in the presence of any concentration of parent PCB 77. As the concentration of parent PCB 77 decreased with increased remediation duration, though, cellular viability improved and superoxide production decreased, as did NFκB activation and mRNA and protein inflammatory marker expression. These data implicate PCB dechlorination as a beneficial tool for decreasing biological toxicity to the vascular endothelium.
Nutrient polyphenol-functionalized nanomaterials were developed to address the need for efficient, environmentally friendly POP remediation. Capitalizing on the previously determined binding affinity of PCBs to aromatic polyphenols, quercetin and curcumin multiacrylates were synthesized and were both surface functionalized onto magnetic iron oxide nanoparticles and incorporated into magnetic nanocomposite microparticle systems. Preliminary results show rapid PCB binding in both MNP and MNM systems, with increased binding contingent on higher polyphenolic polymer content, as seen primarily in MNM binding systems. Additionally, curcumin-based systems exhibited higher PCB binding affinity and higher binding capacity when incorporated into MNM systems. Future work will further substantiate the use of these polyphenolic systems for fluorescence-based, concentration-dependent pollutant sensing.

Finally, green tea extract, comprised primarily of the potent antioxidant polyphenol EGCG, was tested as a nutritional intervention mechanism for PCB-induced \textit{in vivo} toxicity. Mice fed a green tea-enriched diet and subsequently exposed to environmentally relevant doses of coplanar PCB 126 exhibited decreased overall oxidative stress primarily due to the up-regulation of a battery of antioxidant enzymes. F$_2$-isoprostane and its metabolites, established markers of \textit{in vivo} oxidative stress, exhibited fivefold decreased levels in mice fed green tea-supplemented diets. In addition to data indicating increased induction of key antioxidant enzymes, liver mRNA and protein analysis showed that many genes transcriptionally controlled by the aryl hydrocarbon receptor and nuclear factor (erythroid-derived 2)-like 2 proteins were up-regulated in PCB-exposed mice fed the green tea-supplemented diet, which indicates both higher pollutant degradation/excretion and a more efficient antioxidant response to environmental insult. These data support the emerging paradigm that healthful nutrition may be able to bolster and buffer a physiological system against the toxicities of environmental pollutants.

\textbf{5.2 Broad approach to addressing a public health concern}

Overall, this research has sought to address the significant public health risks and enduring remediation challenges posed by PCBs, as well as to further understand potential concerns and opportunities associated with nanomaterial usage. Nutrition is being further substantiated as an important modulator of inflammatory and antioxidant...
pathways, especially associated with environmental insult, and is also emerging as a tool to address exposure toxicity of persistent organic pollutants as both a sensing and remediation platform. Evidence now shows that a person’s nutritional status can play a key role in determining the severity of environmental toxicant-induced pathologies such as diabetes and cardiovascular diseases. People with subprime nutrition may be more susceptible to the toxicity of environmentally persistent pollutants, whereas individuals that make healthy nutritional decisions and emphasize the importance of foods high in bioactive nutrients such as flavonoids may be less vulnerable to environmental insults by being able to buffer against the toxicity of pro-inflammatory pollutants. This research has highlighted that while PCBs have been shown to cause vascular toxicity through ROS-initiated inflammation, the detrimental effects can be counteracted by nutritional modulation via bioactive food components, such as EGCG found in green tea, that have potent antioxidant and anti-inflammatory properties. Thus, the use of healthy nutrition as a tool against the toxicity of environmental pollutants may be an economically and socially viable alternative to expensive and logistically challenging ecological remediation.

Although augmenting the nutritional profiles of at-risk populations may prove to be an effective modulator of toxicant-induced disease in the long term, it is critical to discover and design novel sensing and remediation technologies that are effective, sustainable and environmentally friendly to address immediate needs. To this end, nanoparticle-based remediation systems have been studied to determine new methods for binding and removing organic pollutants from contaminated water sources, biologically-relevant nanoparticle suspension methods have been optimized, and nanotoxicological tests have been performed to understand potential causes of concern. Novel and emerging evidence indicates that bioactive nutrients may be incorporated into nanomaterial-based sensing and remediation platforms for the development of viable technologies that circumvent many of the issues concerning current-day decontamination systems. Ultimately, further research into nutrient polyphenols, such as EGCG, quercetin, and curcumin, will highlight their potential as sustainable, inexpensive components that can both decrease health concerns associated with environmental pollutants in the short term and remediate these concerns in the long term.

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Appendix A. Biomimetic nanocomposite-mediated removal of chlorinated organic pollutants from contaminated water sources

A.1 Synopsis

Polychlorinated biphenyls (PCBs), organic pollutants prevalent in multiple Superfund sites, have proven difficult to remediate due to their high structural stability and their broad environmental presence. Endothelial dysfunction attributed to exposure to PCB contamination may lead to atherosclerosis and steatohepatitis. There is a need to not only address toxicological implications of PCB contamination, but also to detect and remediate contamination sites. Currently, though, there remains an unmet need in the ability to rapidly detect and remove PCBs from a variety of environmental water sources. Ongoing research indicates that polyphenol-functionalized magnetic nanomaterials can serve as a platform for rapid binding of PCBs from contaminated water sources, with eventual use as a combined detection and remediation platform. Quercetin and curcumin antioxidant polyphenols with binding affinities for PCBs were acrylated (producing quercetin multiacrylate (QMA) and curcumin diacrylate (CDA)) and either surface-functionalized onto or polymerized with magnetic iron oxide nanoparticles using PEG200DMA as a co-macromer crosslinker. Polyphenol-functionalized magnetic nanoparticles (MNPs) and magnetic nanocomposite microparticles (MNMs) were characterized for proper functional groups using FTIR (addition of C=O peak at 1740 cm⁻¹), for size using TEM/SEM and DLS (MNPs: approx. 15nm individual particles, 200-400nm aggregates; MNMs: approx. 10µm individual particles, 20 µm aggregates), and for overall composition using TGA (MNPs: approx. 10 wt% polymer; MNMs: approx. 90 wt% polymer). CDA- and QMA-functionalized MNP and MNM PCB binding affinities and capacities were analyzed in 50:50 ethanol:DI H₂O or contaminated groundwater and quantified using LC-MS/MS. PCB binding to materials as a function of binding material concentration, binding time, and as a ratio of polyphenolic polymer mass to bound PCB indicated that MNMs, containing higher percentages of polyphenolic polymer, exhibit higher PCB binding capacities (approx. 80% of initial PCB (5 ppm initial concentration) bound to CDA-MNMs (5 mg/mL)), while optimal PCB binding for all systems occurs within minutes of addition to contaminated water samples. Studies in complex groundwater samples showed that polyphenol-functionalized materials have increased
affinity in comparison to suspended particulate matter (QMA-MNPs captured 87% of PCBs). Initial pollutant release studies indicate a release of 12% bound PCB 126 following AMF heating, which indicates the potential for material regeneration following binding. Overall, quercetin and curcumin incorporation into biomimetic pollutant binding systems significantly increased PCB 126 binding from contaminated water samples and may serve as an effective fluorescence-based pollutant detection system.

A.2 Introduction

More than 30 years after passage of the initial Superfund legislation, risk managers continue to confront high concentrations of persistent chlorinated organic compounds at Superfund sites despite decades of remediation effort. Soil and groundwater aquifer contamination by toxic chlorinated organic compounds at Superfund sites across the country and throughout the world is a pervasive environmental problem which has been studied extensively to further understand associated health consequences as well as to develop effective means of pollutant decontamination and mitigation of negative health effects. For instance, polychlorinated biphenyls (PCBs), persistent organic chemicals heavily used as dielectric and heat transfer fluids prior to their production ban in 1979, have been shown to play a contributory role in a wide variety of chronic diseases such as atherosclerosis, diabetes and obesity. A recent population study by Silverstone et al. identified PCB exposure as a risk factor for development of type 2 diabetes independent of other risk factors, while other studies have shown that circulating levels of PCBs are associated with atherosclerotic plaque formation. PCBs, particularly dioxin-like congeners that are ligands of the aryl hydrocarbon receptor (AhR)(i.e., PCB 77 and 126), have been shown to promote expression of proinflammatory cytokines and to effect changes in redox status, events which act as precursors to these disorders. In addition to highly toxic dioxin-like congeners and despite a total production ban, though, studies have shown that many lesser-known PCBs continue to be produced as unintentional byproducts of pigment manufacturing processes, further increasing exposure risk. The result is that environmentally persistent chlorinated organics present a 21st century challenge.

Biomedical intervention strategies and environmental remediation techniques both have been employed to address environmental pollutant-induced public health concerns. For example, a recently published case study of the residents of Anniston, AL,
a community affected by 30 years of PCB contamination, showed that nutritional intervention with the fat substitute olestra effectively decreased body burden of PCBs. Additionally, green tea extract, primarily composed of the antioxidant polyphenol epigallocatechin gallate (EGCG), has been shown to significantly decrease PCB-induced proinflammatory protein and mRNA markers in vivo by up-regulating a battery of antioxidant enzymes as well as the AhR and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pollutant clearance and antioxidant response mechanisms (see Chapter 5). Other antioxidant polyphenols, i.e., quercetin and curcumin, also have been shown to protect against in vivo and in vitro PCB-induced oxidative stress and have been studied extensively for their use as overall free radical scavengers and as the basis for biodegradable antioxidant polymer systems. While environmental remediation provides a more long-term answer to pollutant-induced public health concerns, this large goal has proven difficult and costly. Traditional treatment strategies, e.g., dredging/soil removal, reactive barriers, six phase heating, microbial transformation, carbon adsorption, etc. for remediation/reclamation of contaminated areas require extensive use of chemicals and/or energy or post-treatment due to formation of undesirable byproducts. Thus, complete remediation has been technically unattainable and economically impractical.

Affinity-based methods (i.e., antibody-based detection systems) are emerging as effective binding and detection platforms for pollutant remediation due to their refined specificity and reduced need for sample purity. Although antibody affinity in the form of enzyme-linked immunoassays currently serves as the most commonly applied immunoassay for environmental PCB detection, antibodies are prone to long-term stability concerns and are expensive to produce. Instead, biomimetic polymeric systems can be tailored that exploit the binding domains of pollutant-specific antibodies while addressing these stability and cost concerns. Studies have shown that binding domains of PCB-specific antibodies (e.g., S2B1) form sterically constrained, highly aromatic pockets that allow for pi-pi bond stacking interactions. This binding relationship is also seen in tests of PCB soil distribution (e.g., humic containing matter) and biodistribution (e.g., lipid rafts, PCB-binding proteins). We hypothesize that synthetic, biomimetic PCB binding domains can be synthesized by incorporating phenolic moieties into polymeric coatings for high PCB binding affinity and capacity as an effective means of pollutant removal from contaminated sites. As with biomedical intervention strategies, polyphenols (e.g., plant derived flavonoids) offer an intriguing
mechanism to address PCB-related public health concerns through their use as precursors for creating biomimetic affinity domains. Similar to the ability of antibodies and natural humic matter to bind PCBs, we hypothesize that nutrient polyphenols (i.e., curcumin and quercetin) can be used as monomeric starting materials to mimic PCB binding microdomains, allowing for the synthesis of PCB binding pockets that enable environmentally-benign pollutant capture/removal systems.

Additionally, the development of magnetic iron-based nanomaterials has brought important and promising new opportunities to the field of contaminant detection and environmental remediation in addition to its wide use for biomedical applications (i.e., magnetic resonance imaging, targeted drug delivery, and hyperthermia)\textsuperscript{40-43}. Beyond their growing use as a platform for PCB dechlorination/remediation\textsuperscript{21; 237; 240; 302; 317-320}, magnetic nanoscale iron particles have attracted growing attention for their easy manipulation with a magnetic field (e.g. for magnetic separation of captured molecules or transport and immobilization at target sites) and response to an alternating magnetic field (AMF) for targeted heating to modify properties (e.g. binding/release properties, reaction rates, etc.). By tailoring nanoparticle surface characteristics through functionalization it is possible to combine the pollutant binding capabilities of biomimetic polymer systems with the separation and heating characteristics of magnetic nanoparticles for effective and environmentally benign pollutant capture/removal from contaminated sites\textsuperscript{41; 42}.

This research employs plant polyphenol-derived polymers incorporated into magnetic nanocomposite platforms that allows for the selective capture and removal of PCB congeners from contaminated water samples (see Figure A.1). This work provides a potential means of environmentally sound pollutant remediation by combining biomedical treatment strategies with materials-based remediation platforms for the creation of biomimetic binding systems with the potential for future use not only in pollutant capture but also detection and release for material reuse.

\textbf{A.3 Materials and Methods}

\textbf{A.3.1 Materials}

Quercetin, curcumin, acryloyl chloride, 2-bromo2-methylpropionic acid (BMPA), iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, dimethyl sulfoxide (DMSO), 2,2'-bipyridine (BPY), ammonium persulfate (APS), and tetramethylethylenediamine
(TEMED) were purchased from Sigma-Aldrich (St. Louis, MO). Triethylamine (TEA), tetrahydrofuran (anhydrous) (THF), acetonitrile (ACN), dichloromethane (DCM), and potassium carbonate ($K_2CO_3$) were purchased from Thermo Fisher Scientific (Waltham, MA). Poly(ethylene glycol) (200) dimethacrylate (PEG200DMA) was purchased from Polysciences (Warrington, PA). 3,3',4,4',5-pentachlorobiphenyl (PCB 126) was purchased from Accustandard (New Haven, CT). All other organic solvents, acids, and salts were obtained from Sigma-Aldrich or Thermo Fisher Scientific and used as received.

### A.3.2 Synthesis of magnetic iron oxide nanoparticles

Magnetic iron oxide nanoparticles were synthesized by a coprecipitation method as previously reported by our laboratory\textsuperscript{40,321}. Briefly, Fe(II) and Fe(III) salts were dissolved in deionized water via sonication at a 1:2 molar ratio, respectively, stirred under $N_2$, and heated to 85 °C. Ammonium hydroxide was added dropwise when the reaction mixture reached 85 °C and was stirred for 1 h to precipitate the nanoparticles. Subsequently, magnetic iron oxide nanoparticles were washed and dialyzed for 24 h in deionized (DI) water. Nanoparticles were characterized for size and suspension stability using dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern, Worcestershire, United Kingdom) (See Table A.1). Particles were stored in DI water until further use.

### A.3.3 Synthesis and purification of quercetin multiacrylate and curcumin diacrylate

Acrylated antioxidant polyphenols were synthesized by reacting the polyphenols quercetin and curcumin with acryloyl chloride in tetrahydrofuran (THF) and potassium carbonate ($K_2CO_3$) or triethylamine (TEA), similar to work previously reported by our laboratory\textsuperscript{128}, to enable their subsequent polymerization (See Supplementary Figure A.1 for synthesis overview). Quercetin and curcumin polyphenols were dissolved in THF at a concentration of 100 mg/mL. $K_2CO_3$ was added to the quercetin solution at a 6:1 $K_2CO_3$:quercetin molar ratio while TEA was added to the curcumin solution at a 3:1 TEA:curcumin molar ratio, followed by purging with $N_2$. Acryloyl chloride was added dropwise to the reaction mixtures with stirring on an ice bath at initial molar ratios (acryloyl chloride:antioxidant) of 6:1 and 3:1 for quercetin and curcumin, respectively. The reactions were allowed to proceed for 18 h at room temperature, protected from light.
Subsequently, THF was removed from acrylated products by vacuum distillation using a liquid N\textsubscript{2} trap, and precipitated salts and potassium bicarbonate were separated by vacuum filtration. In order to remove unreacted acryloyl chloride from the products, dried quercetin multiacrylate (QMA) and curcumin diacrylate (CDA) powders were then redissolved in dichloromethane (DCM) (80 mL) and QMA and CDA were extracted using 0.1 M K\textsubscript{2}CO\textsubscript{3} (3 x 150 mL) and DI water (3 x 120 mL) with centrifugation at 10,000 rpm for 5 min. Residual water was then removed using anhydrous MgSO\textsubscript{4}, solutions were filtered using vacuum filtration, and DCM solvent was removed from purified products under vacuum using a roto-evaporator (Rotovapor R II, Buchi, Switzerland). Synthesized polyphenol multiacrylates were then characterized using high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan). Products were stored at -20 °C until further use.

### A.3.4 Synthesis of polyphenol-functionalized magnetic nanoparticles (MNPs)

Polyphenol-functionalized magnetic iron oxide nanoparticles (QMA- and CDA-MNPs) were synthesized using atom transfer radical polymerization (ATRP) to functionalize acrylates (PEG200DMA and antioxidant multiacrylates) onto BMPA-surface initiated nanoparticles, similar to that reported previously\textsuperscript{322}. Uncoated MNPs were mixed with BMPA initiator at a 1:4 molar ratio in 75:25 water:ethanol (v/v) and stirred for 24 h at RT. The particles were then washed 3x with ethanol, retrieved by magnetic decanting, and suspended at 10 mg/mL in ethanol. Surface initiated magnetic nanoparticles (5 mL in ethanol) were then combined with BPY and CuBr catalysts, along with 2-3 Cu\textsuperscript{0} crystals, at 1:0.01 and 1:0.04 PEG200DMA:catalyst molar ratios, respectively. PEG200DMA was used herein as the primary macromer and QMA/CDA as secondary functional monomer. Catalyst solution was stirred and heated to 50 °C with N\textsubscript{2} bubbling. At 50 °C, 8 mmol macromer (99:1, 95:5, and 90:10 molar ratios of PEG200DMA to QMA or CDA) was injected into the catalyzed mixture and allowed to react for 24 h, protected from light. Functionalized nanoparticles were then magnetically decanted, washed 3x with ethanol to remove excess catalyst, and further washed with 50:50 ACN/DCN, followed by DI water to determine potential macromer leaching. Functionalized MNPs were characterized to determine accurate synthesis characteristics using Fourier transform infrared spectroscopy (FTIR, 7000e, Varian, Palo Alto, CA), for size using DLS and transmission electron microscopy (TEM, 2010F, Jeol, Tokyo, Japan), for polymer weight loss using thermogravimetric analysis (TGA), and for
potential macromer leaching rates using UV-vis spectrophotometry (50 Bio, Varian). Nanoparticles were stored at 4 °C in DI water until further use.

A.3.5 Synthesis of polyphenolic magnetic nanocomposite microparticles (MNMs)

Polyphenolic magnetic nanocomposite microparticles (QMA- and CDA-MNMs) were synthesized by polymerizing primary and secondary macromers in the presence of suspended uncoated magnetic nanoparticles, followed by gel cryomilling to produce particles in micron size regime. PEG200DMA and QMA or CDA were mixed at 99:1, 95:5, and 90:10 molar ratios and polymerized in the presence of 1 wt% magnetic nanoparticles via free radical polymerization with 3 wt% APS and 1.5 wt% TEMED. Following polymer formation in glass templates, gels were cut into small pieces, washed 5x with 50/50 anhydrous ACN/DCN with stirring for 30 min each wash. Solvent was then removed, microparticles were frozen at -20 °C overnight, and subsequently lyophilized overnight. Dried gels were then cryomilled for 10 min using a SPEX 6770 Freezer/Mill (Metuchen, NJ). Polyphenolic MNMs were characterized for size using laser diffraction particle size analysis (SALD-7101, Shimadzu) and scanning electron microscopy (SEM, S-4300, Hitachi, Houston, TX), for polymer weight loss using TGA, and for potential macromer leaching using UV-vis spectrophotometry. Dry MNM powders were stored at -20 °C until further use.

A.3.6 Polyphenol MNP- and MNM-mediated binding of polychlorinated biphenyls from contaminated water samples

PCB 126 was added to 50:50 ethanol:water at 5 ppm to mimic an aqueous environment while the addition of ethanol increases the overall solubility of PCB to enable higher test concentrations. QMA/CDA-MNPs and MNMs were added to PCB-contaminated samples at varying particle concentrations (0.5, 1, and 5 mg/mL) and incubated with shaking at RT for times ranging from 1 min to 2 h to optimize binding conditions. Uncoated magnetic nanoparticles and PEG200DMA-coated MNPs/MNMs served as pollutant binding controls. MNPs/MNMs with bound PCB were magnetically separated and supernatants were removed. Pollutant binding ability and efficiency of the materials were analyzed by measuring remaining PCB in supernatants using a Shimadzu ultra fast liquid chromatography system coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap quadrupole mass spectrometer (LC-MS/MS) in multiple reaction monitoring (MRM) mode using atmospheric pressure chemical ionization (APCI). MRM
transitions monitored: 325.9/256.1, 325.9/254.1, and 325.9/184 for PCB 126. Unbound PCB 126 was analyzed by integrating peak area (area under the curve, AUC) and comparing to that of a concentration curve of known PCB 126 values.

**A.3.7 AMF heating for PCB removal from particles**

The efficiency of PCB 126 release from particle systems following PCB binding was tested to determine the viability of material reuse. An alternating magnetic field (AMF) was applied to heat the polyphenolic MNPs and MNMs for controlled destabilization of the polymer matrix and subsequent release from the particles back in to 50:50 ethanol:water. LC-MS/MS was used as reported in Section A.3.6 to determine PCB leachate concentrations following heating and PCB release.

**A.4 Results and Discussion**

**A.4.1 Material development and characterization**

Polyphenol-functionalized magnetic nanoparticle (MNP) and nanocomposite microparticle (MN) binding systems were successfully synthesized with quercetin and curcumin multiacrylate polymers (QMA and CDA, respectively) to create biomimetic binding systems for chlorinated organic pollutants (i.e., PCBs). First, magnetic iron oxide nanoparticles were produced through a one-pot coprecipitation method as the core material to impart controlled heating and separation functionality. QMA and CDA were then synthesized by incorporating acrylate groups onto the native quercetin and curcumin structures to enable polymerization with poly(ethylene glycol) (PEG), while maintaining original monomer functionality (see reaction scheme, Supplementary Figure A.1). Magnetic nanoparticles were surface-functionalized with QMA and CDA polymers using atom transfer radical polymerization or incorporated within the polyphenolic polymer matrix at 1 wt% and cryomilled to the micron size regime.

In these studies, PEG was used as a crosslinker and co-macromer along with QMA and CDA functional monomers in order to tune the polymer network structure of the polymer coating for tailored PCB binding affinities and matrix porosity. PEG-based systems were selected due to their low non-specific interactions with hydrophobic systems and wide variety of commercially available systems with various molecular weights, allowing for the tuning of polymeric network structures. Following polymerization of CDA or QMA with PEG200DMA to form MNPs and MNMs, FTIR
analysis was used to confirm successful incorporation of desired polyphenolic functional groups, as indicated by the C=O peak observed at 1740 cm\(^{-1}\) (see Figure A.2).

Size analysis of both polyphenolic binding systems (i.e., MNPs and MNMs) was performed using light scattering and electron microscopy methods, as seen in Table A.1 and Figure A.3. TEM analysis of MNPs showed individual particle sizes of 10-20 nm (CDA-MNPs: 11 nm; QMA-MNPs: 15 nm) while DLS showed MNP aggregate hydrodynamic diameters ranging from 240 (CDA-MNPs) to 400 nm (QMA-MNPs). This large increase in particle size is primarily due to the slightly hydrophobic nature of the particles that enables both a higher affinity binding environment for lipophilic PCBs and faster magnetic decantation from contaminated water. Size analysis of cryomilled MNMs showed much larger particle sizes (approx. 10 µm particle sizes via SEM), as expected, and less particle aggregation (aggregate particle diameters of approx. 20 µm via laser diffraction particle sizing).

While nanoparticles exhibit much higher overall surface area than microparticles, with increasing surface area potentially leading to increased total pollutant binding, microparticles are composed of a higher percentage of functional polymer in comparison to the magnetic nanoparticle core material. TGA was used to analyze overall particle composition based on particle mass loss at increased temperatures. As seen in Figure A.4, MNP system mass was attributed primarily to the magnetic nanoparticle core (90 wt% NP core, 10 wt% polymer) while polymeric material predominated in MNM systems (10 wt% NPs, 90 wt% polymer). Leaching studies were also performed to further clarify overall material composition and determine QMA and CDA incorporation rates into the polymeric matrix. MNPs and MNMs were washed following synthesis to remove uncrosslinked macromer and washes were analyzed via UV-vis spectroscopy. Absorbance measured at 370 nm (QMA) or 380 nm (CDA) indicated that CDA was incorporated into both MNP and MNM systems at approx. 90% of initial macromer content while QMA was incorporated at approx. 60-70%.

**A.4.2 Preliminary studies of fluorescence modulation for pollutant detection**

Curcumin and quercetin nutrient polyphenols are of interest not only due to their wide use as natural antioxidant supplements and expected affinity for chlorinated organics, but also due to their inherent fluorescence characteristics that form the basis for future pollutant detection systems and are a key functionality to be exploited with
future experimentation. Preliminary solution phased fluorescence interaction studies were performed to evaluate the potential of quercetin and curcumin esters as fluorescence indicators for PCB interaction. Heterocyclic rings under high concentrations can exhibit pi-pi bond stacking. When the ringed molecules also have fluorescence activity, pi-bond stacking can lead to resonance energy transfer, which shifts the emission spectra into the red\textsuperscript{323;324}. Initially, QMA was tested for potential fluorescence modulation in the presence of PCB 126; when excited at 350 nm, QMA exhibited a fluorescence peak at 390 nm. However, as the concentration of PCB 126 increased from 0.04 to 20 ppm, a second peak emerged at 535 nm, with the ratio between these peaks increasing proportionally over the observed range (see preliminary data in Figure A.5A). Additionally, by characterizing both $K_d$ and potential fluorescence shifts, it is possible to develop a calibration curve for identification of PCB concentration. A Langmuir binding interaction model was used to approximate QMA/PCB binding affinities, indicating a preliminary $K_d$ of the interaction of 2.2 ppm.

While QMA was found to exhibit a PCB-induced fluorescence shift, it was not clear if this capability would remain following incorporation into magnetic nanocomposite binding platforms. Further studies involving QMA-functionalized MNPs were then performed to substantiate the viability of this platform. Surface functionalized MNPs containing 20% QMA were incubated with and without PCB 126 and fluorescence analysis indicated that 6 ppm of PCB resulted in an intense fluorescent peak at 535 nm. This fluorescence shift corresponds directly to that of QMA alone in solution and indicates that QMA incorporation into nanocomposite binding systems does not inhibit binding/sensing activity (see Figure A.5B).

**A.4.3 PCB 126 binding to polyphenol-functionalized nanomaterials**

Capture materials are designed for high affinity of the analyte (i.e., PCB) to the material, the number of available binding sites (binding capacity), and selectivity of the material for the analyte of interest. These binding interactions are the result of multiple weak molecular forces (e.g., pi-pi stacking, electrostatics, hydrogen bonding), which are dictated by the localized environment of the binding pocket. Following substantiation of CDA and QMA affinity for PCBs, CDA- and QMA-functionalized MNPs and MNMs were optimized for binding capacity using a variety of polyphenolic polymer compositions and binding conditions. The magnetic core material of these binding systems enables manipulation with both static and alternating magnetic fields (AMF) for decantation or
remote heating, respectively. As seen in Figure A.6A, MNPs and MNMs well dispersed in medium of interest were completely collected within 5 min following exposure to a static magnetic field. Although the primary purpose of the magnetic core material is to enable simple separation of bound pollutant from contaminated samples, its ability to be manipulated (i.e., remotely heated) with an AMF is key for polymer destabilization for pollutant release. Initial studies performed with magnetic iron oxide nanoparticles at 5 mg/mL showed that temperature increased from 23 °C to 65 °C within 10 min when exposed to an AMF at 300 kHz and 30 kA/m (Figure 4.6B).

Pollutant binding studies were conducted to determine PCB 126 affinity for CDA- and QMA-functionalized MNPs and MNMs as a function of binding material concentration, PCB binding time, and as a ratio of polyphenolic polymer mass to bound PCB. MNPs and MNMs (1 or 5 mg/mL) were added to 50:50 ethanol:water (v/v) containing 5 ppm PCB 126 and incubated with shaking for a variety of time points from 1 min to 2 h. LC-MS/MS analysis was then performed using supernatants following magnetic separation of PCB-bound materials. As seen in Figure A.7A, a five-fold increase in polyphenolic material concentration led to increased PCB binding, although total PCB binding only increased by approx. 20% at higher material concentrations. This indicates that the availability of binding sites plays some role in increased pollutant binding, although equilibrium effects with the 50:50 ethanol:water medium also play some role in incomplete PCB binding. When normalized for polymeric content instead of total particle mass (Figure A.7B), MNPs were shown to bind PCB 126 at much lower rates over the entire study time span. This is both indicative of the primary role that the polyphenolic polymer plays in PCB binding (much increased polymer content in the MNMs yielded significantly higher pollutant binding rates) as well as the role of increased NP surface area in compensating for decreased polymeric content (when comparing PCB binding as a function of total particle mass as opposed to polymeric content). Equilibrium time studies indicated that high pollutant binding is seen within minutes of exposure to functionalized nanomaterials while decreasing pollutant binding, primarily seen with CDA-functionalyzed MNMs, may be due to excessive vortex-based sheer force during pollutant binding.

When transitioning to real-world settings dealing with contaminated groundwater sources, soluble humic acid components (found in sediment and soil samples) have been found to negatively impact PCB sorbent performance. Both competitive binding for
sites on the sorbent material and preferential PCB-humic acid interactions, which may favor the suspension of PCB in the aqueous environment, can lead to decreased binding efficiencies for capture platforms\textsuperscript{325-327}. Herein, preliminary studies were performed to test the viability of polyphenol-functionalized nanomaterials as PCB binding systems in complex media. Groundwater from the Paducah Gaseous Diffusion Plant, Kentucky’s largest Superfund site and the sight of initially significant PCB contamination, (a 50:50 mixture of ethanol to groundwater) was spiked with 5 ppm PCB 126, 1 mg/mL QMA-MNPs were introduced and allowed to bind PCB for 1 h, and bound PCB was magnetically decanted, followed by removal of supernatant. LC-MS/MS analysis indicated that QMA-MNPs captured 87% of PCB 126 in complex media samples, indicating that these polyphenolic functionalities may have higher affinity for PCBs than humic containing matter suspended in water samples (see Figure A.8). This suggests that further development of this system is warranted.

**A.4.4 Reversible capture of PCBs using controlled heating**

Currently, capture materials used for PCB extraction and detection require extensive post processing with large amounts of organic solvents and complex extraction methods. Materials that exhibit switchable affinities for PCBs and other pollutants, though, could provide a means for material reuse without extensive processing with chemicals that also pose health hazards, thus forming a low energy, “greener” approach to pollutant remediation. As highlighted in section A.4.3, magnetic nanomaterials can be heated remotely by applying an AMF to destabilize the polymeric coating material and release PCBs back into the medium of interest. As seen in Figure A.9, QMA-MNPs were used to remove PCB 126 from contaminated water samples, which, following magnetic separation, were resuspended and exposed to an AMF (amplitude of 60 kA/m and frequency of 293 kHz). AMF heating for 10 min led to a 12% PCB 126 release back into the medium (M±SE, n=3) from the MNPs. While it is necessary to further optimize this release system, this ability to remotely impact affinity substantiates further studies for the development of capture/release systems that can easily cycle from low to high affinity binding states.

**A.5 Conclusions**

This research provides a framework for continued experimentation in the development of environmentally benign, biomimetic PCB capture systems. Polyphenolic
macromers based on quercetin and curcumin antioxidant polyphenols with notable affinity for persistent organic pollutants were surface-polymerized onto or polymerized in the presence of magnetic iron oxide nanoparticles to create nanocomposite materials that can be manipulated with a magnetic field. The potential of these systems for fluorescence-based detection of PCBs and as a capture/release platform for material reuse was substantiated. Further, QMA- and CDA-functionalized MNPs and MNMs were shown to bind PCB 126 in both control and real-world contaminated groundwater settings (see Figure A.10 for overall schematic of PCB capture/release platform).

Ongoing experimentation is focused on optimizing material affinities and binding characteristics for a wide variety of PCB congeners, as well as PCB release and material reuse. Future experimentation is needed to further substantiate the use of these materials in fluorescence-based pollutant detection, especially in complex mixtures of organic pollutants, and in higher throughput settings for rapid pollutant binding and contaminated material separation. Overall, this work highlights the use of novel biologically inspired materials for pollutant removal from contaminated water sources and its potential use as a capture/detection/release platform for addressing public health concerns associated with PCBs.
Size analysis performed using dynamic light scattering to determine nanoparticle aggregate hydrodynamic diameters

Size analysis performed using laser diffraction particle analysis to determine microparticle hydrodynamic diameters

**Table A.1**
Particle system size analysis

<table>
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<tr>
<th>Particle system</th>
<th>Analyzed hydrodynamic aggregate size</th>
<th>Analyzed TEM average size (nm)</th>
<th>Analyzed SEM average size (µm)</th>
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<tr>
<td>Uncoated MNPs</td>
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<tr>
<td>PEG-coated MNPs</td>
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<tr>
<td>PEG MNMs</td>
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<tr>
<td>CDA MNMs</td>
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<tr>
<td>QMA MNMs</td>
<td>22.1±0.3 µm&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>11</td>
</tr>
</tbody>
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<sup>a</sup>Size analysis performed using dynamic light scattering to determine nanoparticle aggregate hydrodynamic diameters

<sup>b</sup>Size analysis performed using laser diffraction particle analysis to determine microparticle hydrodynamic diameters
Figure A.1. Overall illustration of composite magnetic nanomaterial platforms for the capture, sensing, and release of chlorinated organic pollutants from contaminated water sources.
Figure A.2. FTIR transmittance showing steps of MNP synthesis. 1) Core magnetic particles are 2) surface initiated by coating with BMPA and 3) functionalized with polyphenolic polymers (i.e., CDA and QMA crosslinked with PEG200DMA). FTIR analysis confirms successful functionalization with the desired polyphenolic polymers via the C=O peak observed at 1740 cm\(^{-1}\), which confirms in this case the presence of quercetin functionality in the polymer coating.
Figure A.3. Electron microscopy particle size determination. SEM analysis of MNMs indicates average particle sizes of A) QMA MNMs, B) CDA MNMs, and C) PEG MNMs as 11, 10, and 8 \( \mu m \), respectively. TEM analysis of MNPs indicates average particle sizes of D) QMA MNPs and E) CDA MNPs as 11 and 15 nm, respectively.
Figure A.4. Thermogravimetric analysis of functionalized nanomaterial systems. Weight-loss profiles were analyzed to determine average polymer content of MNP and MNM binding systems, indicating that MNMs were composed of approximately 90% polymeric material and 10% magnetic nanoparticles. Conversely, approximately 90% of the mass of MNPs is due to the core magnetic nanoparticles while polyphenolic polymer surface coating comprises 10% overall particle mass.
Figure A.5. Fluorescence-based sensing of PCB 126. QMA and PCB demonstrate a concentration dependent fluorescence shift. A) When excited at 350 nm, QMA exhibits a fluorescence peak at 390 nm. As PCB 126 increased from 0.04 to 20 ppm, a second peak emerged at 535 nm, with the ratio between these peaks increasing proportionally over the observed range. Using a Langmuir binding interaction model to approximate QMA/PCB binding affinities it was found the $K_d$ of this interaction as 2.2 ppm. B) Studies involving QMA-functionalized MNPs also showed a concentration specific fluorescence peak at 535 nm upon QMA-MNP/PCB binding.
Figure A.6. Material response to static and alternating magnetic fields. A) MNPs and MNMs can be completely separated from PCB-contaminated water samples within 5 min after exposure to a static magnetic field. B) Illustration of the heating of Blank MNPs in an alternating magnetic field (Amp = 60 kA/m, Freq = 293 kHz) (M±SE, n=3).
Figure A.7. PCB 126 capture by polyphenolic MNPs and MNMs. CDA- and QMA-functionalized MNP and MNM binding affinities for PCB 126 (5 ppm) were analyzed as a function of binding material concentration, PCB binding time, and as a ratio of polyphenolic polymer mass to bound PCB. A) a five-fold increase in polyphenolic material concentration leads to increased PCB binding, although total PCB binding only increased by approx. 20% at higher material concentrations. B) When normalized for polymeric content instead of total particle mass, MNPs were shown to bind PCB 126 at much lower rates over the entire study time span (M±SE, n=3). This is both indicative of the primary role that the polyphenolic polymer plays in PCB binding (increased polymer content yields higher pollutant binding) as well as the role of increased NP surface area in compensating for decreased polymeric content. Equilibrium time studies indicate that
high pollutant binding is seen within minutes of exposure to functionalized nanomaterials while decreasing pollutant binding, primarily seen with CDA-functionalized MNMs, may be due to excessive vortex-based sheer force during pollutant binding.
Figure A.8. QMA-MNP capture of PCB 126 from contaminated groundwater samples. Contaminated water samples from the Paducah Gaseous Diffusion Plant were spiked with 5 ppm PCB 126, 1 mg/mL QMA-MNPs with varying QMA content was introduced into samples, and remaining PCB 126 in supernatant following magnetic separation was analyzed with LC-MS/MS. Functionalized particles capture 87% of PCB in complex media samples, indicating that material binding affinities are higher than that of the particulate matter suspended in the water samples (M±SE, n=3).
Figure A.9. Temperature-induced PCB 126 release from MNPs. QMA-MNPs were heated with AMF (amplitude of 60 kA/m and frequency of 293 kHz), which led to a 12% PCB 126 release back in to supernatant (M±SE, n=3).
Figure A.10. Overall schematic of PCB capture and material regeneration. Polyphenol-functionalized materials (MNPs or MNMs) can be introduced into PCB-contaminated water sources within a closed system where PCBs bind within biomimetic binding pockets. The application of a static magnetic field can then be used to magnetically decant PCB-bound MNPs and MNMs, leaving decontaminated groundwater, while exposure to an alternating magnetic field can serve to destabilize the polymer matrix, releasing bound PCB to be further remediated while also regenerating the polyphenolic material for reuse.
Appendix B. Additional data

Supplementary Figure 2.1. Transmission electron microscopy images of characteristic alumina aggregates under various conditions. (A) Alumina dispersed in citrate buffer, pH 6.0, with 10 min sonication using a probe sonicator showed mean aggregate diameter of ~72 nm, while alumina suspended using ultrasonic bath with (B) 24-h sonication and (C) 6-h sonication resulted in aggregate diameters of ~84 nm and 113 nm, respectively. Average aggregate sizes of citrate-stabilized alumina suspended in (D) DMEM/High Glucose medium and (E) F-12K medium remain relatively the same, with average particle diameters of ~93 nm and ~79 nm, respectively. Although primarily suspended at sizes below 100 nm, some alumina particle agglomeration was seen when suspended in media that resulted in particle diameters of ~150 nm. Mean aggregate diameter represents n≥5 TEM data.
## Supplementary Table 4.1
Experimental diets

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<th>1% GTE-supplemented diet</th>
<th>Control diet</th>
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<tr>
<td></td>
<td>g/kg</td>
<td>kcal</td>
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<tr>
<td>Protein</td>
<td>19 (20%)</td>
<td>19 (20%)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>67 (70%)</td>
<td>67 (70%)</td>
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<tr>
<td>Fat</td>
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<td>4 (10%)</td>
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<tr>
<td>Casein</td>
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<tr>
<td>L-Cystine</td>
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<td>Corn Starch</td>
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<tr>
<td>Maltodextrin 10</td>
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<tr>
<td>Green Tea Extract</td>
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<tr>
<td><strong>Total</strong></td>
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<td>4057</td>
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Supplementary Figure 4.1. PCB 126 exposure leads to a significant increase in liver/body weight ratio (hepatosomatic index, HSI) in both control and GTE-supplemented diets, although GTE supplementation did not significantly mitigate this PCB-induced increase. Statistical analysis was performed using two-way ANOVA followed by Tukey test with *p<0.001. A trend toward a statistically significant interaction between diet and PCB 126 treatment (p=0.06) was also seen.
Supplementary Figure 4.2. Relative mRNA levels of antioxidant enzyme markers. Overall, GTE supplementation did not significantly increase antioxidant mRNA levels in control diets, but, in the presence of environmental perturbation (i.e., PCB 126 gavage), significantly higher antioxidant levels were seen in mouse liver above non-supplemented diet. All values were determined using the relative quantification method ($\Delta\Delta Ct$) as a fold change from control. Vehicle and PCB 126-treated mice with 10% fat control and GTE-supplemented diets: n=8-10. Data are presented as mean±S.E.M (*p<0.01, **p<0.05). See Table 5.2 and Figure 5.3 for more information concerning antioxidant markers tested.
Supplementary Figure A.1. Curcumin diacrylate (CDA) and quercetin multiacrylate (QMA) synthesis schemes. To enable crosslinking with PEG200DMA, A) curcumin and B) quercetin were first acrylated to form CDA and QMA, respectively, as seen in the above reaction mechanisms. These polyphenols were reacted with acryloyl chloride to form ester bonds between the acid chloride (–OCl) of acryloyl chloride and the polyphenol hydroxyls (–OH), with the release of HCl byproduct that is neutralized with K$_2$CO$_3$. 
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