THE APPLICATION OF METABOLOMICS TO THE EVALUATION OF THE CELLULAR TOXICITY

A Thesis

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Master of Science

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Metabolomics is an emerging field that measures all small molecule species within the cell and their changes in response to genetic reprogramming, environmental toxin exposure, and disease. Currently, the assessment of cell toxicity induced by exposure to environmental contaminants as well as nanomaterials is mainly limited to the measurement of overt cell death in tissue culture or animals. The rapid profiling of multiple biochemical species by liquid chromatography/mass spectrometry (LC/MS) based metabolomics allows the elucidation of early molecular toxicity with one technological platform. In this thesis, we assess the cell toxicity of nanomaterials, polystyrene and L-tyrosine polyphosphate (LTP) as well as two common indoor environmental contaminants, sodium hypochlorite and benzalkonium chloride vapor in the macrophage cell line, THP-1. Utilizing a number of bioinformatics programs including XCMS online, it was found that cells exposed to nanomaterials show upregulation of the lysophosphocholine, 1-heptadecanoyl-sn-glycero-3-phosphocholine which may function as a putative marker of increased fatty acid uptake.
I wish to show my appreciation to Dr. Jun Hu. Dr. Hu guided me to find a new procedure to prepare 2,4,9-trithiaadamantane-7-carboxylic acid methyl ester. I really like this research experience. Every day we discussed the potential mechanisms for the reaction, came up with new ideas, kept testing and killing them quickly, and finally we got the new methodology. Also thanks for his generous sharing about his invaluable life experience, which may be much more important than academic skills for me.

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CHAPTER I
INTRODUCTION

1.1 Mass spectrometry-based metabolomics

1.1.1 Metabolomics

The central dogma of biochemistry states that within eukaryotic cells, DNA is transcribed to mRNA and mRNA is translated into proteins.\(^1\) Many of these proteins catalyze catabolic and anabolic reactions that are involved in signal transduction, biomass synthesis, and energy production. The metabolites in these reactions in total compose the metabolome of a cell or organism. These reactions form a complex network of events; therefore, it is necessary to detect the complete set of metabolites to determine the metabolic response of cells to physical or chemical stimulation.

Metabolomics provides a powerful method to directly connect genotype to phenotype at the molecular level.\(^2\) Other technologies such as genomics, transcriptomics and proteomics are upstream of cellular metabolism. In contrast, metabolomics is a direct read-out of cellular phenotype as metabolites are influenced by both gene and protein expression. The combination of systems biology platforms may reveal new functions for known genes and proteins.\(^3\)\(^-\)\(^5\) In addition, combining metabolite detection with isotope tracers has allowed the identification of altered metabolic pathways in disease that represent novel drug targets.\(^6\)\(^-\)\(^8\)
Detecting metabolite changes associated with gene mutations or diseases has long been used in medicine. Alkaptonuria and phenylketonuria are two examples of inborn errors of metabolism, and cause tyrosine and phenylalanine metabolism dysregulation respectively. Increased metabolites at the point in the metabolic pathway where loss of function occurs can be detected in urine or blood as a biomarker. One disadvantage of these clinical analyses is that only one or a few metabolites are examined from each sample. Quantitative analysis of a large numbers of metabolites followed by computerized pattern recognition was subsequently developed to yield valuable information on unknown pathways that may be involved in disease processes. Evaluation of global metabolism holds promise to provide a better understanding of the mechanisms of disease along with potential biomarkers.

A second field where metabolomics is being applied is in nutritional studies. Scientists are beginning to study the relationship between diet and metabolism to allow quantitative analysis of how diet could be altered to impact metabolism. For example, to explore the putative suppressive mechanisms of whole-grain cereals on the development of type 2 diabetes and heart disease, individuals with metabolic syndrome were offered an oat, wheat bread, and potato (OWP) diet or a rye bread and pasta (RP) diet for 12 weeks. Serum metabolic profiles of the two groups were analyzed. Compared with the RP group, the OWP group showed a series of increased lysophosphatidylcholine species, which are related to oxidative stress and inflammatory effects. In another example, plasma lipid profiles from a diet-induced weight reduction group and a control group of obese individuals were determined. The diet-induced weight loss primarily decreased the levels of saturated triacylglycerol (TG) species, especially for those
containing short chain fatty acids, while sphingolipid and lysophosphatidylcholine levels didn’t change.

1.1.2 Liquid chromatography methods

Liquid chromatography (LC) is one of the most powerful and widely used techniques to separate mixtures. The system consists of a column that contains a stationary phase and solvents used for the mobile phase. Modern columns are typically composed of silica particles that can be bonded with chemical groups whose characteristics determine the properties of the column. Mobile phases can be water or other organic solvents. Separation in LC is based on the interaction of compounds with the column and the mobile phase and this is determined by compound properties such as polarity and size. Compounds interacting for a shorter time with the stationary phase will elute sooner than those interacting for a longer period of time, this leads to efficient separation of chemically diverse species.

High-performance liquid chromatography (HPLC) works by the same principles as classical column chromatography with regard to theory; however, new instrumentation such as a high-pressure pumping system to control the rate and composition of the mobile phase, autosamplers, more robust stationary phases, highly sensitive detectors and a computer system to control the overall process allow separation of compounds at higher pressure and reduced times.\textsuperscript{14} A major advantage of separation before mass spectrometry (MS) analysis is a reduction in ion suppression caused by co-eluting compounds. Therefore, LC prior to introduction of sample into the mass spectrometer results in better detection limits and data quality.
Reversed phase liquid chromatography (RPLC) is a commonly used chromatographic method utilized in metabolomics. The stationary phases of RPLC is chemically bonded with nonpolar groups such as C\textsubscript{8}, C\textsubscript{18}, C\textsubscript{30} alkyl chains.\textsuperscript{15,16} The mobile phase is typically a mixture of water and acetonitrile (ACN) or methanol (MeOH), due to the compatibility of these two organic solvents with ultraviolet (UV) detection and their low viscosity. The initial concentration of water is usually high, resulting in a polar mobile phase and as the gradient progresses the percentage of organic solvent is increased to elute hydrophobic compounds. RPLC is used to analyze neutral samples or those polar samples in neutral form.\textsuperscript{17} The polar solutes are retained less due to the fact that they partition in the polar mobile phase. These compounds will be eluted out of the column first. Nonpolar solutes interact strongly with the nonpolar stationary phase by partition and absorption and they will leave the the column last.\textsuperscript{18} In short, solutes will leave the column in order of decreasing polarity.

The resolution of the RPLC can be adjusted by changing the composition of the mobile phase. Choosing the proper concentration of water can optimize retention time and resolution, because increasing organic solvent concentration will decrease the retention time of different solutes by different degrees.\textsuperscript{19} In some cases only a three-component ACN/water/MeOH mobile phase can lead to the maximum resolution.\textsuperscript{20}

While RPLC provides efficient separation of hydrophobic metabolites, polar compounds exhibit poor retention on RPLC. The mobile phase of hydrophilic interaction chromatography (HILIC) is similar to RPLC, a mixture of water and acetonitrile. However, its stationary phases is similar to normal-phase chromatography with high polarity and includes silica, amine, and diol.\textsuperscript{21} This allows the polar analytes that are
normally not retained in RPLC to be separated with HILIC. In contrast to RPLC, the retention time in HILIC can be decreased as the polarity of mobile phase increases. The elution gradient for HILIC begins with a low water percentage in the mobile phase for separation of hydrophobic compounds. As the run continues, the percentage of water is increased to allow polar compounds to be eluted. This makes HILC a complementary technique to RPLC for global metabolomic profiling.\textsuperscript{22} In order to increase the ionization efficiency of analytes as well as pH of the mobile phase, ammonium acetate or formate are commonly included.\textsuperscript{23} Temperature can also influence separation efficiency depending on the stationary phase. For silica type-A columns, a higher column temperature will decrease retention time but increase selectivity and column efficiency, while for silica type-B columns the situation is just the opposite.\textsuperscript{24} Although the mechanism of HILIC is still not well understood, it is commonly accepted as a combination of partitioning and ion exchange. A water layer is thought to form on the surface of the stationary phase. The polar analytes partition into this layer and charged polar analytes also undergo ion exchange with the charged silica molecules.\textsuperscript{25} Because of this combination mechanism, HILIC can be applied to polar neutral, basic and acidic analytes.

The performances of several different HILIC and RPLC columns were tested in a non-targeted and a targeted manner for urinary metabolic profiling.\textsuperscript{26} HILIC columns had improved performance, especially the diol-HILIC column, due to detection of increased numbers of molecular features and the reduction of analysis time. In a second study, HILIC and RPLC columns were used to profile healthy and fatty liver tissues. Increased
lipid biomarkers were identified with RPLC while HILIC separated amino acids more efficiently.\textsuperscript{27}

1.1.3 Mass spectrometry methods

Metabolomics should be able to identify and quantify as many metabolites as possible, because low abundance metabolites may have biological activity. MS-based detection of metabolites provides the necessary sensitivity and specificity to identify perturbations in diverse cellular pathways. In contrast to other analytical approaches such as NMR, MS-based metabolomics detects large numbers of compounds simultaneously. Within the field of MS-based metabolomics there are two types of analytical approaches: targeted metabolite analysis and untargeted metabolomic analysis, or global metabolite profiling.\textsuperscript{28,29}

Targeted methods focus on qualitative and quantitative characterization of known metabolites in a series of pathways such as those metabolites involved in amino acid or nucleotide metabolism. If profiling is combined with isotope tracers, this type of targeted profiling can elucidate the concentration change of small-molecule precursors as cells undergo alterations in their physiology such as when there is rapid replication or during viral infection. For example, the core metabolic pathways during embryogenesis of \textit{X. laevis} were investigated.\textsuperscript{30} Those metabolites whose abundances changed during early embryonic development were detected by LC/MS, after cells were fed with compounds containing stable isotopes. Metabolic tracing of these compounds demonstrated that alanine was consumed to generate energy for the early embryo and that nucleotide levels were exhausted at the onset of the mid-blastula transition, which could trigger
lengthening of the cell cycle. A second study investigated metabolic changes that accompany viral replication with the hope that perturbed pathways may provide novel targets for anti-viral drugs. Flux analysis was performed in human fibroblasts infected with human cytomegalovirus (HMCV). This study revealed that among 41 fluxes examined, 28 changes occurred between the uninfected and infected cells, including the nucleotide biosynthesis pathway, which is a current target for the treatment with HCMV infection. The research also showed that fatty acid biosynthesis is increased in HCMV-infected cells similar to nucleotide biosynthesis, suggesting that inhibitors of fatty acid biosynthesis could be used to impair HCMV replication.6 This was proven by inhibiting viral replication with 5-tetradecyloxy-2-furoic acid (TOFA), an inhibitor of acetyl-CoA carboxylase an enzyme critical for fatty acid biosynthesis.31 Metabolomics was also used to investigate HCMV with another related virus, herpes simplex virus type-1 (HSV-1). Infection of fibroblast and epithelial cells by each virus triggered strong metabolic changes which were similar across different host cell types; however, HCMV mainly affects lipid metabolism while HSV-1 mainly alters deoxypyrimidine metabolism and increases the flux through upper glycolysis.8 To further investigate a biological role for individual enzymes in the specific mechanism of HSV-1 infection, an siRNA screen of metabolic genes was used and argininosuccinatesynthetase 1 (AS1) was identified as an important influence on HSV-1 replication.32 This enzyme consumes aspartate as part of de novo arginine synthesis. By using LC/MS targeted metabolomics, it was shown that the metabolic phenotype induced by knockdown of AS1 in human fibroblasts mimicked the phenotype of HSV-1 infection, including an increase in multiple nucleotides and their
precursors. These results suggested that HSV-1 decreased AS1 protein levels to aid its own replication.

In contrast to a targeted approach where only known metabolites of interest were observed, global metabolomic analysis is used to provide a comprehensive analysis of metabolic reactions in total and detect as many metabolites as possible without bias. This approach places more value on the discovery of novel metabolites, which can provide clues to an unknown role of a specific gene or protein. For example, in the known synthesis pathway of the iron chelating small molecule, pyochelin (pch), by the bacteria *Pseudomonas aeruginosa* two novel metabolites were detected by untargeted metabolomics. P. aeruginosa secretes pyochelin for the acquisition of iron from the surrounding environment. The synthesis of this molecule is catalyzed by a series of enzymes encoded by the *pch* gene cluster. The wild-type and mutants of a series of *pch* genes strains were cultured and extracted. Apart from pyochelin, the levels of two other metabolites were decreased markedly in the mutant strains and were characterized as 2-alkyl-4,5-dihydrothiazole-4-carboxylates by a combination of mass spectrometry, chemical synthesis, and stable isotope labeling. Subsequent assays confirmed that their production is dependent on iron level and the *pchE* gene in the *pch* gene cluster. Another study examined whether an orphan nuclear receptor, neuron-derived clone 77 (Nur77), has a functional ligand binding site and uses ligand-binding to control transcription. LC/MS-based untargeted metabolomics was used to detect protein-metabolite interactions and identify the ligands. Like other nuclear receptors, Nur77 has a ligand binding domain (LBD) at the C terminal region. A vector containing a polyhistidine tag and the gene for the LBD of Nur77 was used to express the His6-tagged Nur77 ligand-
binding domain (Nur77LBD). Putative ligands were then identified by immobilizing this construct and incubating with a lipid mixture extracted from brain or testes where Nur77 is expressed. It was found that the column containing Nur77LBD enriched only unsaturated fatty acids (UFAs).

Untargeted metabolomics also holds great promise for identifying metabolites associated with specific diseases. For example, lipid levels were monitored in cells replicating human hepatitis B virus.7 One overexpressed lipid was identified as 7-dehydrocholesterol (7-DHC), which provides strong motivation for further studies characterizing the functional role of 7-DHC in infection. In another example, global metabolic changes in plasma and cerebrospinal fluid (CSF) were compared from three groups of individuals with Alzheimer’s Disease (AD), mild cognitive impairment (MCI) or normal cognition (CN).33 Multiple changes in neurotransmitter metabolism and energy metabolism related to mitochondrial dysfunction and lipid biosynthesis were detected, consistent with previous studies of these conditions.34,35 Among many variables, lysine in plasma was the best biomarker for both MCI and AD, while prostaglandin 2 in CSF can be used to differentiate MCI from AD. Another study sought to identify a reliable diagnostic method for the neurodegenerative disease, amyotrophic lateral sclerosis (ALS), in CSF. A group of metabolite variables was integrated into a multivariate model, which predicted more than 80% cases. However, in this study four significant variables still could not be identified due to technological limitations of LC/MS.36
1.1.4 Bioinformatics

Bioinformatics is an interdisciplinary field that combines biology, statistics, mathematics and computer science. High-throughput bioanalytical technologies, such as gene sequencing, proteomics, and metabolomics generate a huge amount of data. In order to mine useful information out of the data, it is necessary to employ efficient and accurate computational methods and software.\(^{37}\)

Within the discipline of LC/MS-based metabolomics, the challenge lies in the analysis of large numbers of compounds that are capable of being detected by the mass spectrometer. In this analysis each compound is identified by a mass-to-charge ratio ($m/z$) and retention time. However, in order to determine the biological relevance of up- or down-regulated metabolites it is necessary to find those features that can best distinguish between two groups. There are two types of the analysis: unsupervised methods to determine the groups and supervised methods to confirm the important variables.\(^{38}\) In supervised methods, samples of test and control groups have been differentiated before analysis, and the variables which can distinguish the groups are determined and used to classify new samples. In contrast, the samples are not labeled before unsupervised analysis and unsupervised methods will try to find those variables which best distinguish samples and then classify samples into different groups based on these variables.

Principal Components Analysis (PCA) is an unsupervised technique for multivariate analysis.\(^{39}\) This analysis is commonly used to reduce a dataset that contains a large amount of variables to one that is smaller without losing the information contained within the large set. The initial dataset containing test and control groups are imported as a matrix. The number of the compounds detected in each sample is on the order of $10^3$
and the intensity of each compound can be utilized as one dimension to identify the sample, so the matrix is a high-dimensional space and every sample corresponds to a point within that space. By rotating the axis, one new axis, the first principal component (PC1), lies along the line of maximum variance, and each sample can be projected onto this new axis and given a PC1 score. During the projection each compound in the sample has different loadings, and the larger loading represents the larger importance in distinguishing between the groups. PC1 explains the greatest amount of variance; PC2 explains the next largest amount and so on.

The t-test is used to determine how well a metabolite level change distinguishes the test and control group. The t-test calculates the p-value and t-value for each metabolite variable and the smaller p-value, the higher the probability that the metabolite level change is significant. The t-value can be compared with a calculated critical value. A t-value larger than the critical value means the corresponding metabolite level change does distinguish the two groups.

XCMS Online is a web-based platform to process untargeted metabolomics data generated by LC/MS. Column degradation, fluctuations in the column temperature, mobile phase pH deviations and other variations can lead to retention time drifts from sample to sample, which should be compensated for. These fluctuations require data processing for retention time correction before statistical analysis. XCMS online also performs peak picking and alignment to accurately find peaks that can be compared between different samples.

After the peak picking and alignment, the platform will determine the charge state and adducts of the precursor ion automatically. The charge state of each compound is
determined based on the spacing of the isotope peaks. If charge is successfully assigned and this peak has the lowest \(m/z\) value of the isotope cluster, the peak is determined to be monoisotopic. The adduct ions are determined based on the spacing of the precursor ion and the nearby characteristic peak with lower \(m/z\) value.

XCMS online incorporates automated PCA analysis and Welch’s t-test. The scores plot contains a point for each sample. The separation between two groups of points is determined by differences in detected peaks between samples. The results of the t-test are visualized by the cloud plot or a box and whisker plot for individual metabolites.

To obtain the preliminary identity of a compound of interest the accurate mass of the compound is first searched in metabolite databases: METLIN (Metabolite and Tandem MS Database, http://metlin.scripps.edu/) and HMDB (Human Metabolome Database, http://www.hmdb.ca/). METLIN is a metabolite database created and maintained by the Center for Mass Spectrometry at the Scripps Research Institute. It contains masses, chemical formulas and structures for over 64,000 endogenous and exogenous metabolites. The database provides their MS/MS patterns at different collision energies for more than 10,000 metabolites. This database is coupled with XCMS online, which further facilitates metabolite identification. HMDB is another widely-used metabolite database. It contains 41,514 metabolites, which are linked to 5,688 protein and DNA sequences and contains related chemical or clinical data.

One \(m/z\) value of a precursor can correspond to several possible molecular formulas, and each molecular formula can also corresponds to numerous potential isomers. To correctly identify features, matching of MS/MS fragmentation patterns is performed manually by inspection. Although many metabolite databases have included a
large number of compounds, the number is still small compared with metabolites detected from biological samples and many detected features do not return any matches. In addition, techniques to determine the stereochemistry of the isomers as well as the positions of double bonds within lipids are limited with current high-throughput approaches.

When both the accurate mass and the fragmentation data of a metabolite peak are matched, standards should be run on the same instrument with the same instrument parameters. When metabolites are not available commercially, chemical synthesis is the only choice. The retention time and fragmentation patterns must match between the sample and the standard.

1.2 Evaluation of cellular toxicity

The determination of nanotoxicity involves a number of parameters including overt cytotoxicity, perturbations in organ function, biodistribution, and clearance. Within each of these categories multiple tests are commonly utilized to determine whether the materials induce overt cell death or disrupt essential functions. These assays provide a picture of potentially adverse interactions between organisms and nanomaterials.

Cytotoxicity assays provide a binary read-out of cell death and several methods are available that allow quantitative assessment of nanoparticle toxicity. Trypan blue is a cheap and effective method to measure cell viability, based on the principle that living cells will exclude the dye.44

Another widely-used method employs the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye. This compound is a water-soluble tetrazolium salt
that is yellow in its natural state. When MTT enters into living cells it passes into the mitochondria and the tetrazole ring is disrupted after reduction by mitochondrial succinate dehydrogenase, forming a purple-colored, insoluble formazan compound (Figure 1.1). Measuring the absorbance at 570 nm reflects the number of viable cells in the culture.45

![Figure 1.1 Reduction reaction of MTT in mitochondria.](image)

For whole organ toxicity, distribution of nanomaterials in different organs is the first concern.46 One study examined the biodistribution of 10 nm cerium oxide nanoparticles injected into mice intravenously or intraperitoneally for 2 or 5 weeks. Tissue sections of major organs were then prepared and viewed for nanoparticle deposition by high resolution transmission electron microscopy and histopathology. Cerium levels were measured by inductively coupled plasma mass spectroscopy.47 The nanoparticles were found mainly in the spleen and liver, two organs of the reticuloendothelial system. These results suggested that macrophage endocytosis was an important factor for the distribution of nanoceria. In another study, the size effect on distribution of gold nanoparticles (AuNPs) was investigated. AuNPs from 15 nm to 200 nm were administered to mice intravenously. Smaller size particles showed increased
internalization and deposition in all tissues. Surprisingly 15 and 50 nm AuNPs were able to pass the blood-brain barrier and accumulate in brain tissue.\textsuperscript{48}

There have been several preclinical assays to assess drug-induced liver injury for candidate compounds screening, including measuring levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP).\textsuperscript{49} The most frequently used hepatotoxicity indicator is serum alanine aminotransferase (ALT) activity level. ALT catalyzes the reductive transfer of an amino group from alanine. ALT can be found in many tissues such as liver, skeletal muscle and heart tissue, and its release into blood is associated with organ injury. However, this assay may show false positives when used for the evaluation of hepatotoxicity.\textsuperscript{50} Some newer biomarkers for hepatotoxicity with greater sensitivity and specificity are in development. Sorbitol dehydrogenase (SDH) catalyzes the reversible oxidation-reduction of sorbitol. SDH enzymatic activities correlate broadly with general types of liver injury like ALT. However, the stability of the SDH assay needs to be improved, since the SDH in frozen serum is only stable for three days.\textsuperscript{51} Arginase I, serum F protein and glutathione-S-transferase alpha (GST\textalpha) are more sensitive and more liver-specific injury markers than ALT. Arginase I is a hydrolase primarily localized to liver that catalyzes the transformation of arginine to urea and ornithine, and shows high liver specificity.\textsuperscript{52} Serum F protein is a dioxygenase which catalyzes the conversion of 4-hydroxyphenylpyruvate into homogentisate.\textsuperscript{53} GST\textalpha catalyzes the conjugation of glutathione with reactive metabolites.\textsuperscript{54} In addition, serum F protein and GST are only localized to liver and kidney. However these newer biomarkers are currently measured by ELISA-based assays. This leads to a high cost associated with the use of the corresponding antibodies.\textsuperscript{55}
1.2.1 THP-1 cell line

Monocytes and macrophages are phagocytic cells that are part of the innate immune system. Monocytes circulate in blood and move into tissues as part of normal surveillance or in response to inflammatory signals where they differentiate into macrophages. A major function of macrophages is to kill invading pathogens. The pattern recognition receptor proteins on the surface of macrophages recognize highly conserved macromolecules of viruses or bacteria, which then induce macrophages to engulf and destroy these pathogens. Macrophages also produce cytokines and chemokines to potentiate innate responses and promote adaptive immune responses.

The THP-1 cell line is a human acute monocytic leukemia cell line established in 1980 that serves as an in vitro model of macrophage function. The THP-1 cell line provides several advantages as a research model. They display minimal variability in the cell phenotype due to their homogeneous genetic background which contributes to less variance in experimental situations. The cells are easily subcultured and stored in liquid nitrogen. THP-1 cells are round suspension cells, with distinct monocytic markers: the line expresses complement (C3) and Fc (the fragment crystallizable region of an antibody) receptors, which mediate monocyte adhesion to infected cells, and lack surface or cytoplasmic immunoglobulin.

After phorbol-12-myristate-13-acetate (PMA, also referred to as TPA) treatment, THP-1 cells stop proliferating, become flat with irregular shapes, adhere to the flask wall, and differentiated into mature cells that function as macrophages. After differentiation these cells still express Fc receptors and lysozyme activity, but α-naphthyl butyrate esterase is enhanced and the cells are able to phagocytize yeast and immunoglobulin G-
coated sheep erythrocytes. PMA-treated THP-1 cells express a series of proinflammatory cytokines, including interleukin-8 (IL-8), macrophage chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). These cytokines are involved in numerous aspects of the immune response to infection. IL-8 and MCP-1 induce neutrophils and monocytes to migrate from blood to the tissues. TNF-α can trigger blood clotting in the local small vessels to prevent the pathogen spreading through blood. TNF-α, IL-1, and IL-6 also produce various long-range defense effects, including elevation of body temperature, induction of acute-phase proteins and leukocytosis.

THP-1 cells have been used to examine the involvement of macrophages in disease pathology. For example, these cells have been used to explore the mechanisms of atherosclerosis. THP-1 cells acquire the capacity to produce lipoprotein lipase (LPL) after initiation of PMA-induced differentiation and this is in contrast to other leukemia cells such as HL60 and U937. LPL is responsible for the hydrolysis of the core triglycerides in triglyceride-rich lipoproteins to glycerol and free fatty acids and plays a role in the development of atherosclerotic plaques. The PMA-induced differentiation of THP-1 is also accompanied by an increase other proteins, including the ApoE scavenger receptor and high density lipoprotein receptor. All these changes lead to an enhanced capacity to accumulate cellular cholesterol and form foam cells.

Another study evaluated the extent to which THP-1 cells mirror monocytes-macrophages in the vasculature by determining interactions between THP-1 cells and various vascular cells, as well as the transcriptome, microRNA profile, and histone modifications of THP-1 cells in response to various inflammatory stimuli. THP-1 and
primary cells exhibit differences in protein and mRNA expression that should be controlled strictly when using THP-1 as a model. With respect to cholesterol ester metabolism, the triglyceride (TG) content of THP-1 cells incubated with acetylated LDL is much higher than the cholesterol ester (CE) content, which is opposite to the lipid composition of foam cells in vivo. An acyl-CoA synthetase inhibitor should be used to decrease TG content in PMA-induced THP-1 cells, so they can mimic foam cells in this aspect.  

In addition to their role in cholesterol metabolism and vasculature function, macrophages are also “first-responders” during infection. Lipopolysaccharide (LPS) derived from Gram-negative bacteria can activate macrophages to participate in the inflammatory immune responses. Engagement of LPS to toll-like receptor 4 (TLR4) activates NF-κB and which leads to the release of proinflammatory cytokines, including IL-1, IL-6, and TNF-α.  

TNF-α plays important role in several inflammatory diseases such as narcolepsy, multiple sclerosis and Alzheimer’s disease. This factor was suggested as a possible target for therapy in these diseases. PMA-induced THP-1 have the ability to secrete TNF-α in response LPS stimulation. THP-1 also display a similar phenotype to primary rat microglia and can be used to investigate the signaling pathways involved in inflammatory responses during Alzheimer’s disease.

1.2.2 Metabolomics for the evaluation of nanoparticle toxicity

The classical toxicity assays are convenient and simple, but they often do not provide information about molecular alterations that occur in response to toxins. More comprehensive analysis of inflammatory responses as well as alterations in metabolism
that may precede overt organ injury is needed to completely evaluate the safety of materials. For example, the cytotoxicity of sodium fluoride (NaF), a preventive agent used in dentistry has been analyzed. A series of target metabolites were measured and the results suggested that NaF may stimulate the onset of glycolysis, but inhibit the enolase reaction and TCA cycle progression. ATP utilization was identified as an apoptosis marker, while oxidized metabolites were regarded as non-specific markers regardless of whether cells were undergoing apoptosis or necrosis. This platform was also developed to predict cytotoxicity of drugs by measurement of 50-100 targeted metabolites.

Currently humans are often exposed to many nanomaterials which are defined as materials of 100 nanometers or less, such as the silica found in toothpastes, titanium oxide found in cosmetic products, and nanoparticles that are being developed for drug delivery and imaging. The size and large surface area-to-volume ratio allows more potential reactive groups on the particle surface to interact with biological tissue. The link between ultrafine particle exposure and disease is known and some examples includes silicosis and asbestosis. Although the mechanisms of toxicity are still being investigated for many materials, it is thought that ultrafine particles cannot be completely digested by macrophages, leading to the generation of reactive oxygen species (ROS) and lysis. In addition, these particles may activate the Nalp3 inflammasome leading to release of cytokines such as IL-1 and IL-18 that stimulate fibroblasts to form fibrous tissue. Nanoparticle exposures that lead to excess ROS production may also overwhelm the natural antioxidant defenses. This results in activation of pro-inflammatory signaling cascades leading to inflammation. Inflammation will further perturb mitochondrial function and stimulate the release of proapoptotic factors, which finally results in cellular
apoptosis or necrosis.\textsuperscript{81} The specific mechanism and extent of ROS generation strongly depends on the chemical composition of the material and its surface groups.

Two materials that warrant investigation for potential cellular toxicity are polystyrene and polymeric nanoparticles. Polystyrene, a synthetic polymer (Figure 1.2), has been tested for its potential application in drug delivery\textsuperscript{82} or as a biomaterial to implant into the human body.\textsuperscript{83,84} Biocompatibility and toxicity are concerns when polystyrene contacts blood and human tissue \textit{in vivo}.

![Chemical structure of polystyrene](image)

\begin{center}
\textbf{Figure 1.2 Chemical structure of polystyrene.}
\end{center}

L-tyrosine polyphosphate (LTP) is a synthetic polymer (Figure 1.3), that was developed by Dr. Yang H Yun’s group. LTP nanoparticles have previously been explored as a potential nonviral gene vector for gene therapy with low toxicity, prolonged degradation and proper release rate.\textsuperscript{85} The potential to induce inflammatory and innate immune system responses was examined by comparing NO production from bone marrow cells exposed to LTP nanoparticles and LPS.\textsuperscript{86} No significant activation of the innate immune system was observed; however, since these particles were derived from amino acids, we sought to examine whether cellular metabolism, for example, amino acid metabolism and TCA cycle, may be perturbed after internalization of these nanoparticles.
Figure 1.3 Chemical structure of L-tyrosine polyphosphate.

In this thesis, untargeted metabolomics was used to detect metabolic changes induced in THP-1 cells by interactions with a variety of synthetic materials including polystyrene, tyrosine polyphosphate nanoparticles, and household contaminants. These metabolites may be biomarkers of early toxic or inflammatory changes that could be monitored during safety studies.
CHAPTER II
MATERIALS AND METHODS

2.1 Materials

The THP-1 cell line was obtained from Dr. Sailaja Paruchuri’s group, Department of Chemistry, University of Akron. RPMI 1640 media was obtained from Mediatech. Standard fetal bovine serum and penicillin-streptomycin (10 mg/mL) were obtained from VWR. The bleach used in these experiments was Tandil® ultra liquid bleach, with sodium hypochlorite as the active ingredient; the Lysol used was Lysol® All Purpose Cleaner Trigger, with benzalkonium chloride as the active ingredient. LTP nanoparticles were obtained from Dr. Yun Yang’s group, Department of Biomedical Engineering, University of Akron. Two polystyrene standard samples PS1780 (M_w=1780, M_n=1630, PDI=1.09) and PS201 (M_w=201, M_n=197, PDI=1.02) were obtained from Scientific Polymer and the laboratory of Dr. Chrys Wesdemiotis, Department of Chemistry, University of Akron. PMA was purchased from Sigma Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Acros. Bicinchoninic Acid (BCA) Protein Assay was obtained from G-Biosciences. LC solvents including methanol, acetone, and acetonitrile were LC/MS grad and purchased from Fisher Scientific. Distilled, deionized water was obtained from MillporeSynergy® Water Purification Systems.
2.2 Sample Preparation

2.2.1 Cell culture and drug treatment

THP-1 monocytes were grown in suspension at 37°C and 5% CO\textsubscript{2} in RPMI containing 10% fetal bovine serum and 100 µg/mL penicillin-streptomycin. The cells were subcultured in another flask every 3 to 5 days when the cell density reached near $10^7$ cells/mL. The cells were counted under microscopic observation using a haemocytometer.

Polystyrene treatment: The THP-1 cells were exposed to PS1780 in DMSO solution, PS201 in DMSO solution and pure PS201 separately. For each experiment, $5 \times 10^5$ THP-1 cells in 2 mL of the culture medium per well were distributed into two 6-well plates (one as the treatment group and the other as a control group). The polystyrene suspension was prepared as follows. PS1780 was an insoluble solid at room temperature in water. A 1.2 mg sample of PS1780 and 300 µL DMSO were mixed in a 15 mL centrifuge tube and oscillated rapidly in a vortex mixer for 1 minute. Then 6 mL of the culture medium was added into the tube and the mixture was vortexed again for 1 minute. For the treatment group, 1 mL of polystyrene suspension was added to each well. The cells were allowed to grow in a 5% CO\textsubscript{2} incubator at 37°C for 3 days. Cells were harvested into 15 mL centrifuge tubes and spun down at 1000 rpm for 10 minutes. The medium was discarded and the pellet of each tube was washed with 1 mL PBS. Samples were transferred to 1.5 mL Eppendorf tubes, pelleted again at 1000 rpm, and stored at -80°C until extraction.

Control cells for the PS1780 and PS201 treatment were treated with 300 µL DMSO in 6 mL culture medium. For the pure PS201 treatment, the polystyrene
suspension consisted of 1.2 mg of PS201 and 6 mL culture medium, and fresh tissue culture medium was used for the control.

LTP nanoparticles treatment: $1 \times 10^6$ THP-1 cells/well were plated overnight followed by the addition of 1 mL of a 0.27 mg/mL stock solution of LTP nanoparticles to each well. Controls received fresh tissue culture medium. The cells were allowed to grow in a 5% CO$_2$ incubator at 37°C for 6 hours prior to metabolite extraction.

Bleach and Lysol treatment: 7.2 µL of 5 mM PMA was dissolved into 12 mL of the culture medium and vortexed for 1 minute and each well received 1 mL of PMA solution to induce differentiation. The cells were allowed to grow in a 5% CO$_2$ incubator at 37°C for three days until cells became adherent to the tissue culture plate.

Prior to bleach or Lysol vapor treatment, new media was added and the test plate was covered by a Pyrex container next to a beaker containing about 20 mL of bleach or Lysol. The bottom was covered with aluminum foil. Cells were then incubated in 5% CO$_2$ incubator at 37°C for another 3 days. After 3 days, the metabolites were extracted from cells.

2.2.2 Metabolite extraction

600µL of cold MeOH was added to each tube containing a cell pellet. Samples were vortexed for 30 seconds, frozen in liquid nitrogen, and thawed at room temperature for 3 minutes. The solutions were sonicated for 1 minute and this cycle was repeated three times. Samples were incubated at -20°C for 1 hour, and then centrifuged at 13000 rpm at 4°C for 10 minutes. The supernatant was transferred to a new Eppendorf tube, while 400 µL of cold acetone of -20°C was added to the pellet. The pellet and acetone
mixtures were vortexed for 30 seconds and sonicated for 1 minute followed by incubation at -20°C for 30 minutes. Samples were centrifuged for 10 minutes at 13000 rpm at 4°C. The two supernatant solutions were combined and concentrated. The solids contained the small-molecule metabolites and were stored at -80°C, while the remaining pellets contained the proteins. Pelleted proteins were retained in order to determine extraction efficiency with the BCA protein assay.

The metabolites were normalized to protein content and samples were redissolved in 95% ACN/water and sonicated for 10 minutes. Then the solutions were kept 4°C for 30 minutes and centrifuged 10 minutes at 13000 rpm at 4°C. The supernatants were transferred into LC vials and kept at -80°C until LC/MS analysis.

2.3 LC/MS method

Liquid chromatography was performed using a HILIC column (Luna 3 μm NH₂ 100 Å, LC Column 250 × 4.6 mm) on a Eksigent Ekspert™ microLC 200 system with mobile phases consisting of water with 5 mM ammonium acetate and acetonitrile with 5mM ammonium acetate. The elution gradient is shown in Table 2.1. The gradient started at 0% water with a 4 minute linear gradient to 20% acetonitrile, followed by an 8 minute linear gradient to 100% acetonitrile. The total flow rate was 20 μL/min, and the injection volume was 5 μL.
Table 2.1 Gradient elution program used for the HILIC-MS.

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Samples were analyzed on the AB SCIEX TripleTOF® 5600 System with Duospray Ion Source. The nebulizer gas (N₂) pressure was 15 psi and the drying gas pressure was 20 psi. The drying gas temperature was 300 °C. The capillary voltage was set at 5 kV and the collision energy to 40 V. The TOF and MS2 mass range were 60-1000 Da and 10-1000 Da.
CHAPTER III
RESULT AND DISCUSSION

In order to uncover pathways associated with toxicity, we performed a comparative analysis between cells exposed to nanomaterials as well as environmental contaminants and controls. Our workflow for these experiments is detailed in Figure 3.1 A. Small molecules were extracted from cells using organic solvents and separated with HILIC prior to their introduction into the mass spectrometer. Data was subsequently collected and analyzed with XCMS online in order to determine whether THP-1 cells were responding to exposure of these materials by altering their metabolism (Figure 3.1 B). A representative total ion chromatograph is shown in Figure 3.1 C: in the left window, the X axis is the retention time for LC, and the Y axis is the molecular weight from 100 to 1000 and individual metabolites obtained in the right window.
Figure 3.1 Instrumentation set-up for LC/MS-based metabolomics. (A) Schematic diagram of LC/MS system, (B) Schematic diagram of data generating process and (C) the raw data displayed in the Analyst® Software.
To ensure statistically significant correlations, appropriate sample sizes were selected with the typical sample size being 4-10 replicates per cell culture. In our experiments, each group was composed of six samples. The amount of metabolites extracted from each sample should be large enough to meet the detection limits of the LC/MS platform and an aliquot of 1 million cells provides sufficient signal-to-noise to determine metabolites of biological interest.

Before XCMS online performs peak picking and alignment, the appropriate parameters for the LC/MS system should be chosen depending on the LC/MS instrument and methods. In this thesis, for feature detection, the maximal tolerated $m/z$ deviation was 50 ppm and the chromatographic peak width was from 5 sec to 100 sec; for retention time correction, the step size (in $m/z$) was 1; for alignment, the width of overlapping $m/z$ slices was 0.015, the minimum fraction of samples was 0.5 and the allowable retention time deviations was 5 sec. The statistical analysis was performed with a p-value set to less than 0.01 and a fold change greater than 2.0. Putative adducts identified included $+H$, $+NH_4$, $+Na$, $+K$ with a tolerance of 100 ppm for the database searches. We set the tolerance high to ensure that we would capture endogenous metabolites since the currently available databases often contain a large number of xenobiotic agents and plant metabolites.

For example, Figure 3.2 A shows the original total ion chromatograms (TICs) obtained from comparing cells treated with sodium hypochlorite to controls. XCMS Online performs automated retention time correction allowing overlay of chromatograms from multiple LC runs. XCMS online also displays retention time deviation to allow an evaluation of the chromatographic method.
Figure 3.2 XCMS Online processing of metabolomic data. (A) Total ion chromatograms from control samples and those exposed to bleach before correction. Colored lines are individual samples. (B) The retention time deviation and (C) the corrected, overlaid total ion chromatograms. After correction peak matching allows a comparison between sample groups.
3.1 Influence of polystyrene on cell metabolism

In three experiments, THP-1 cell lines were exposed to polystyrene for three days. Two polystyrene samples with different molecular weights, PS1780 and PS201, were used. In the first experiment, the high molecular weight PS1780 was an insoluble solid in the culture medium, so it was dissolved in DMSO first. When the DMSO solution plus PS1780 were added into the aqueous culture medium, PS1780 precipitated out again as very fine small particles; therefore, it is possible that there was little interaction of the larger polystyrene particulates with the cells. In all three experiments, no clear changes in appearance of the cells were observed after three days and experimental cells were similar to controls although there was some evidence of cell swelling in a few samples. Since there was little change in cell shape that might indicate necrosis or apoptosis, we hypothesized that metabolomic analysis might detect early alterations in cells in response to the materials that might be influenced by molecular weight. For the cells exposed to PS1780 in DMSO, PCA analysis showed the treatment groups (red dots) and control group (blue dots) were separated only in the PC1 direction (Figure 3.3 A). The lack of separation indicates few metabolic changes in response to treatment and indicated that PS1780 displayed relatively low levels of toxicity or limited interaction with the cells.

For the cells exposed to PS201 in DMSO, PCA analysis showed larger separation between the treatment groups and the control samples in PC1 direction (Figure 3.4 A). Finally the cells exposed to pure PS201 showed the largest separation (Figure 3.5A). These results demonstrate that smaller molecular weight polystyrene molecules may be more easily internalized by THP-1 cells leading to greater changes in cellular metabolism. Significant features were identified and visualized using a cloud plot. We set the p-value
≤ 0.01 and the fold change ≥ 2 for the t-test. There were 580 features detected for the cells exposed to pure PS201 (Figure 3.5 B), compared with 329 features for the exposure to PS201 in DMSO (Figure 3.4 B) and 124 features for PS1780 in DMSO treated cells (Figure 3.3 B).

Figure 3.3 Statistical analysis of THP-1 cells treated with PS1780 and DMSO. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). (B) Cloud plot showing results of the Welch’s t-test. The plot shows TICs for each sample with retention time along the x-axis and m/z along the y-axis. Green circles indicate up-regulated metabolites and the size indicates fold change while red circles are down-regulated metabolites. Most metabolites of dysregulated cluster at the later retention times indicating dysregulation of polar metabolites.
Figure 3.4 Statistical analysis of THP-1 cells treated with PS201 and DMSO. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). (B) Cloud plot showing results of the Welch’s t-test. The plot shows TICs for each sample with retention time along the x-axis and $m/z$ along the y-axis. Green circles indicate up-regulated metabolites and the size indicates fold change while red circles are down-regulated metabolites.

Figure 3.5 Statistical analysis of THP-1 cells treated with PS201. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). (B) Cloud plot of showing results of the Welch’s t-test. The plot shows TICs for each sample with retention time along the x-axis and $m/z$ along the y-axis. Green circles indicate up-regulated metabolites and the size indicates fold change while red circles are down-regulated metabolites.
The total numbers of features detected in the PS1780/DMSO, PS201/DMSO, and pure PS201 experiments were 2450, 3948 and 3139 respectively. Ten metabolites with significantly changed abundances and good peak shapes were targeted for identification using fragmentation information (Table 3.1). Their precursor masses and MS/MS fragmentation patterns were searched in two metabolite databases: HMDB and METLIN. Current metabolomic databases are incomplete with regards to mammalian metabolism and often lack MS/MS spectra for compounds. With this limitation in mind, we identified a feature, with \( m/z \) of 510.1 and a retention time of 9.3 minutes which was identified as 1-heptadecanoyl-sn-glycero-3-phosphocholine (Figure 3.6 A). Both the accurate mass and the fragmentation data of a metabolite peak match well (Figure 3.6 B). 1-heptadecanoyl-sn-glycero-3-phosphocholine (LPC 17:0) is a lysolecithin, resulting from partial hydrolysis of phosphatidylcholines. The acyl group of the LPC 17:0 is heptadecanoic acid which is in low concentrations in humans but found in higher amounts in ruminants. This feature was detected in the exposure to PS201 in DMSO with a fold change of 6.1 and in the exposure to pure PS201 with a fold change of 11.7 (Figure 3.7), but not detected in the exposure to PS1780 in DMSO. Therefore, upregulation of phospholipids containing this fatty acid may indicate increased fatty acid uptake from serum containing tissue culture medium by polystyrene-stimulated macrophages.
Table 3.1 Metabolites altered in response to polystyrene treatment. (A) Treatment with pure PS201. (B) Treatment with PS201 in DMSO. (C) Treatment with PS1780.

(A)

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(B)

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Figure 3.6 Preliminary identification of 1-heptadecanoyl-sn-glycero-3-phosphocholine. (A) Structure of the compound. (B) MS/MS spectra showing the characteristic fragment at $m/z =$ 184 of the phosphocholine head group.
Figure 3.7 Upregulation of 1-heptadecanoyl-sn-glycero-3-phosphocholine after exposure to PS201/DMSO and pure PS201. (A) Extracted ion chromatograms for the exposure to pure PS201 (treatment group indicated with red lines and control group as black lines). (B) Box and whisker plot of LPC 17:0 levels in THP-1 cells treated with PS201 vs. controls (treatment group at the right side and control group at the left side). (C) Extracted ion chromatograms for the exposure to PS201/DMSO. (D) Box and whisker plot of LPC 17:0 levels in THP-1 cells treated with PS201/DMSO. Lower levels are seen in control cells vs. PS201 treated cells.
3.2 Influence of LTP nanoparticles on cell metabolism

In order to understand potential changes in cellular physiology that occur after exposure of macrophages to LTP nanoparticles, we performed an untargeted metabolomics analysis. No alterations were detected in appearance of the cells after the exposure. The PCA scores plot of LTP nanoparticles treated samples and controls is shown in Figure 3.8 A. Little separation of the experimental and control groups were seen on PCA, indicating that cells showed similar metabolism (Figure 3.8 B). The t-test indicated that nineteen metabolites were dysregulated with nine metabolites significantly increased and ten metabolites decreased relative to control. Metabolites with significantly changed abundance and good peak shapes were targeted for identification using fragmentation patterns (Table 3.2). No matches were determined for most metabolites, but the variable with $m/z = 510.4$ and retention time of 9.3 minutes, was also seen to be upregulated in these samples (Figure 3.9). These results suggest that uptake of significant amounts of polymeric material containing tyrosine does not impact metabolism.

Table 3.2 Metabolites altered in response to LTP nanoparticles.

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Figure 3.8 Statistical analysis of THP-1 cells treated with LTP nanoparticles. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). (B) Cloud plot showing results of the Welch’s t-test. The plot shows TICs for each sample with retention time along the x-axis and m/z along the y-axis. Green circles indicate up-regulated metabolites and the size indicates fold change while red circles are down-regulated metabolites. Few metabolites show significant alterations in response to LTP treatment.

Figure 3.9 Upregulation of 1-heptadecanoyl-sn-glycero-3-phosphocholine after exposure to LTP nanoparticles. (A) Extracted ion chromatograms for the exposure to LTP nanoparticles (treatment group indicated with red lines and control group as black lines). (B) Box and whisker plot of LPC 17:0 levels in THP-1 cells treated with LTP nanoparticles vs. controls (treatment group at the right side and control group at the left side).
3.3 Influence of sodium hypochlorite and benzylalkonium chloride on cell metabolism

There is increasing concern that individuals may experience adverse health effects due to excessive exposure to indoor contaminants. Humans are exposed to a variety of volatile organic compounds from sources such as construction or decoration materials, new furniture, paint, and various cleaners. Sodium hypochlorite (NaClO) and benzylalkonium chloride are the active ingredients of commonly used volatile cleaners, bleach and Lysol respectively. We sought to evaluate whether exposure to volatiles common in the indoor environment could induce inflammatory metabolic changes.

Cells treated with bleach vapor were extracted and metabolites analyzed with our untargeted platform. PCA scores plot showed PC2 separated the control samples from the test groups, but there was no separation in PC1 direction (Figure 3.9 A). This suggests that changes in global metabolism are small and this was confirmed by examining fold changes and p-values for detected features (Figure 3.9 B). In total, only 35 metabolite changes were detected. For the treatment with Lysol vapor, there was not complete separation in PC1 and PC2, similar to results with sodium hypochlorite (Figure 3.10 A). However, in contrast to sodium hypochlorite, 151 metabolite changes were detected (Figure 3.10 B). These results suggest that Lysol vapor may induce increased dysregulation of metabolism.

For the two experiments, metabolites with significantly changed abundance and good peak shapes were targeted for identification using fragmentation patterns, but no matching compounds were obtained (Table 3.3 and 3.4). We expected that there might be variable glutathione (GSH), which functions in antioxidant defenses. Oxidative stress has been shown to decrease levels of GSH leading to the accumulation of oxidized
glutathione\textsuperscript{81}. However, obvious alterations in glutathione concentration were not detected in these experiments.

Figure 3.10 Analysis of THP-1 cell lines treated with sodium hypochlorite vapor. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). Little separation of the groups is evident. (B) Cloud plot showing the 35 features dysregulated in response to exposure to bleach vapor.

Figure 3.11 Analysis of THP-1 cell lines treated with benzalkonium chloride vapor. (A) PCA analysis of the treatment group (blue spots) and the control group (red spots). Little separation of the groups is evident. (B) Cloud plot showing the 151 features dysregulated in response to exposure to benzalkonium chloride vapor.
Table 3.3 Metabolites altered in response to sodium hypochlorite vapor treatment.

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Table 3.4 Metabolites altered in response to benzalkonium chloride vapor.

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3.4 Conclusion

Metabolomic analysis of macrophages exposed to both polymeric materials and environmental contaminants indicates that these materials can induce alterations in basal cellular metabolism. Upregulation of LPC 17:0 appeared to indicate an increased uptake of fatty acids from the environment; other unidentified metabolites may shed further light
on whether exposure to particles or volatiles can induce inflammatory or protective pathways. Compared with PS201 treatment, PS1780 treatment led to fewer metabolite changes. The reason is still not clear. One explanation is that molecular weight influences metabolism. A second explanation was that THP-1 cells did not engulf PS1780 as irregular solid particles with large sizes as well as PS201 as liquid droplets. To clarify the problem, future experiments will focus on testing 50 nm PS1780 nanoparticles.\(^8\) It has been shown that cells effectively engulf this particle size.\(^4\) Comparing the 50 nm PS1780 treatment with PS201 treatment may provide evidence that higher molecular weight particles have reduced cellular uptake.

The experiments with sodium hypochlorite and benzalkonium chloride demonstrate that chemical vapor exposure has the potential to change cellular physiology and these pathways may serve as markers for levels of indoor pollution. The air pollutants caused by sodium hypochlorite can include gaseous chlorine (Cl\(_2\)) and hypochlorous acid (HOCl),\(^8\) but the amounts of pollutants generated by sodium hypochlorite were small enough in normal conditions that household bleach vapor is considered nontoxic.\(^9\) Human periodontal ligament cells were cultured in medium containing NaClO. The results of an MTT assay showed that the NaOCl mainly inhibited the mitochondrial activity, while no protein inhibition could be observed using \([^3H]\)-leucine monitoring.\(^9\) Our results were in accord with the previous studies and provided more detail about metabolite changes caused by NaClO. Benzalkonium chloride has been added in eye drops as a preservative and its nontoxic dose was determined as 0.001% (weight/volume).\(^9\) However, even 0.00001% benzalkonium chloride stimulated
phagocytosis, migration, and cytokine release by PMA-treated THP-1. Future studies will identify dysregulated metabolites and link them to inflammatory cytokine production.
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