In vitro and in vivo toxicological evaluation of emissions from the fused filament fabrication three-dimensional printing

Mariana T. Farcas
mfarcas@mix.wvu.edu

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Part of the Environmental Health Commons, Occupational Health and Industrial Hygiene Commons, and the Toxicology Commons

Recommended Citation
Farcas, Mariana T., "In vitro and in vivo toxicological evaluation of emissions from the fused filament fabrication three-dimensional printing" (2021). Graduate Theses, Dissertations, and Problem Reports. 8106. https://researchrepository.wvu.edu/etd/8106

This Dissertation is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself. This Dissertation has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.
In vitro and in vivo toxicological evaluation of emissions from the fused filament fabrication three-dimensional printing

Mariana T. Farcas

Dissertation submitted
to the School of Pharmacy
at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor in Philosophy in
Pharmaceutical and Pharmacological Sciences

Yong Qian, Ph.D., Advisor
Yon Rojanasakul, Ph.D., Chair
Vincent Castranova, Ph.D.
Timothy R. Nurkiewicz, Ph.D.
Aleksandr B. Stefaniak, Ph.D.

Department of Pharmaceutical Sciences

Morgantown, West Virginia
2021

Keywords: 3-D printing, emissions, inhalation, toxicity evaluation, in vitro, in vivo, NHBES, ALI.

Copyright 2021 Mariana T. Farcas
ABSTRACT

In vitro and in vivo toxicological evaluation of emissions from the fused filament fabrication three-dimensional printing

Mariana T. Farcas

Fused filament fabrication (FFF), a three-dimensional (3-D) printing process, is an emerging technology that has recently gained wide popularity among both consumers and manufacturers. As filament is heated to above its glass transition temperature in a 3-D printer, a portion may undergo thermal decomposition, which releases ultrafine particles (UFP) and volatile organic compounds (VOCs) with potential adverse respiratory health effects are released into the air. This study's central hypotheses is that emissions generated during 3-D printing are toxic and exposure to these emissions induces pulmonary and systemic adverse health effects. Considering that currently, limited understanding is available on the health impact of the FFF 3-D printer exposures, the overall goal of this research was to fill the knowledge gap by pursuing the following three studies.

The aim of the first study was to assess acrylonitrile butadiene styrene (ABS) and polycarbonate (PC) filaments 3-D printer emissions-induced cell toxicity. In this study, the particles and vapors released during printing were collected directly into the cell culture medium and delivered to the small airway epithelial cells at six concentrations. At 24 h, various endpoints, such as cellular uptake, cell viability, cell membrane damage, ROS production, total antioxidant capacity, glutathione peroxidase levels in cell lysates, cell death mechanisms (apoptosis and necrosis), and cytokines and chemokines released in cell supernatants were measured. To analyze the data, mixed model regression analyses were performed on these endpoints using particle numbers as the independent variable. The regression lines illustrated a significant dose-response relationship between a decrease in cell viability and the number of emitted particles, which correlated with a significant dose-dependent increase in the LDH activity, and all other endpoints evaluated.

The aim of the second study was to assess pulmonary and systemic toxicity in rats following whole-body inhalation exposure to ABS filament 3-D printer emissions. In this study, male Sprague Dawley rats were exposed to filtered air or ABS emissions for 1, 4, 8, 15, or 30 days (4 h/day, 4 days/week). The average mass concentration and number of particulate generated during 4-h real-time printing of three desktop 3-D printers operating simultaneously was 240 µg/m³ and 8.84 x 10⁴ particles/m³. At the start of the exposure (day 1), a predominant pro-inflammatory response was seen in BALF, represented by an increase in IFN-γ and TNF-α Th1-type cytokines followed by a switch to an anti-inflammatory response by day 15 of exposure represented by a rise in IL-10 Th2-type cytokine. The Th1/Th2 switch could be responsible for the initial “delayed” influx of the alveolar macrophages and its peak occurrence at 15 days of exposure, which corresponded with a significant increase in blood monocytes and platelet counts. Other systemic changes noted were that initially (day 1), a significant increase in both hepatic and renal biomarkers was found; however, at day 15 of exposure, only renal biomarkers were increased. At the longest exposure duration (day 30), all the endpoints evaluated returned to the control levels. Neither pulmonary oxidative stress responses nor histopathological changes of the lungs and nasal passages were found among the treatments.

The aim of the third study was to evaluate the pulmonary effects of FFF 3-D printer emissions using a relevant human ALI organotypic airway tissue model to mimic the respiratory behavior upon exposure. Primary normal, human-derived bronchial epithelial cells (NHBEs) were directly exposed for 4 h to ABS filament emissions. NHBEs epithelium integrity and differentiation changes, cytotoxicity, tissue injury, and inflammatory and immune system regulation markers were evaluated following exposure and 24 h after the end of the exposure. Overall, at the conditions applied, exposure of NHBEs to ABS emissions did not affect epithelium integrity,
ciliation, mucus production, or induce cytotoxicity. At 24 h after the exposure, significant increases in IL-12p70, IFN-γ, TNF-α, IL-17A, VEGF, and MIP-1α were noted in the basal cell culture medium of ABS-exposed cells compared to chamber control cells.

Overall, toxicological evaluation of FFF 3-D printer emissions was conducted in three different conditions, 1) traditional submerged culture of human small airway epithelial cells, 2) repeated whole-body inhalation exposure of rats to freshly generated aerosols, and 3) advanced human ALI organotypic airway tissue model. At the experimental conditions applied, in both in vitro models and in vivo, 3-D printer emissions produced minimal to moderate pulmonary toxicity. Furthermore, these findings were consistent with results observed in the physiologically relevant in vivo-like in vitro model cultured at ALI. In conclusion, these studies indicate that the FFF 3-D printer emissions could induce moderate toxicological effects. These studies are significant as they are amongst the first and comprehensive studies published to evaluate the pulmonary and systemic toxicity of 3-D printer emissions. Further studies are needed to establish a more broad exposure-dose-response relationships and integration of in vivo and in vitro responses.
DEDICATION

To my dear husband and children.
ACKNOWLEDGEMENTS

I would like to express my most profound appreciation to my committee members. First and foremost, I am deeply grateful to my advisor and mentor, Dr. Qian. My success would not have been possible without your dedicated support, wisdom, and encouragement. I would like to extend my gratitude to the experts' committee members. Thank you, Dr. Rojanasakul, Dr. Vincent Castranova, Dr. Timothy R. Nurkiewicz, and Dr. Aleksandr B. Stefaniak, for your enthusiastic assistance and guidance throughout my research project. I am very grateful for your insightful feedback and suggestions after each committee meeting!

My gratitude extends to the National Institute for Occupational Safety and Health (NIOSH) and the U.S. Consumer Product Safety Commission (CPSC) for the funding opportunity to undertake my studies.

I am grateful to all of those with whom I have had the pleasure to work during this project. Thank you, Alycia Knepp, Lauren Bowers, Dr. Marlene Orandle, Dr. Kyle Mandler, Dr. Todd Stueckle, Dr. Jayme Coyle, Lori Battelli, Sherri Friend, Dr. Kristine Krajnak, and Walter McKinney! Without your passionate participation, this work could not have been successfully conducted.

I would also like to thank Janet Thompson, Dr. Nicole Olgun and Dr. Vamsi Kodali for their friendship, helping me survive all the stress, and not letting me give up.

Most importantly, my appreciation also goes out to my family for their encouragement and support in the pursuit of my goal. I would like to thank my parents, sister, and brother, whose love and guidance are with me in whatever I follow. Mom and dad, thank you for allowing me to pursue the career I wish for since I was in 7th grade. My dear grandparents, I miss you so much! I miss your hugs and the time we spent gardening. I wish I could share this achievement with you. Most importantly, I am grateful to my loving and supportive husband, Daniel, and our three restless children. Daniel, thank you so much for caring for the kids and me when I was occupied.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>I</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>II</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>IV</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>V</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>VI</td>
</tr>
<tr>
<td>LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE</td>
<td>IX</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>1.1. ADDITIVE MANUFACTURING</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1. Fused filament fabrication</td>
<td>7</td>
</tr>
<tr>
<td>1.1.2. Thermoplastics used in fused filament fabrication</td>
<td>8</td>
</tr>
<tr>
<td>1.1.2.1. Manufacturing and uses of Acrylonitrile-Butadiene-Styrene</td>
<td>9</td>
</tr>
<tr>
<td>1.1.2.2. Manufacturing and uses of Polycarbonate</td>
<td>10</td>
</tr>
<tr>
<td>1.2. FUSED FILAMENT FABRICATION EMISSIONS CHARACTERIZATION</td>
<td>11</td>
</tr>
<tr>
<td>1.2.1. Nanoparticles</td>
<td>11</td>
</tr>
<tr>
<td>1.2.2. Volatile organic compounds</td>
<td>23</td>
</tr>
<tr>
<td>1.3. HEALTH AND SAFETY CONSIDERATION OF FUSED FILAMENT FABRICATION 3-D PRINTER EMISSIONS</td>
<td>28</td>
</tr>
<tr>
<td>1.3.1. Nanoparticles</td>
<td>28</td>
</tr>
<tr>
<td>1.3.1.1. Deposition behavior of nanoparticles emitted from 3-D printers</td>
<td>28</td>
</tr>
<tr>
<td>1.3.1.2. Mechanisms of nanoparticle induced-toxicity</td>
<td>33</td>
</tr>
<tr>
<td>1.3.1.2.1. Oxidative stress: concept and biomarkers</td>
<td>33</td>
</tr>
<tr>
<td>1.3.1.2.2. Inflammation: concept and biomarkers</td>
<td>35</td>
</tr>
<tr>
<td>1.3.1.2.3. Translocation: concept and biomarkers of systemic toxicity</td>
<td>38</td>
</tr>
<tr>
<td>1.3.2. Volatile organic compounds</td>
<td>42</td>
</tr>
<tr>
<td>1.3.3. FFF 3-D printer emissions and printed parts toxicity studies: a literature review</td>
<td>47</td>
</tr>
<tr>
<td>1.3.3.1. In vitro studies</td>
<td>47</td>
</tr>
<tr>
<td>1.3.3.2. In vivo studies</td>
<td>48</td>
</tr>
<tr>
<td>1.3.3.3. Human studies</td>
<td>48</td>
</tr>
<tr>
<td>1.4. RESEARCH GAPS AND PURPOSE OF THE STUDY</td>
<td>49</td>
</tr>
<tr>
<td>1.5. CONCEPTUAL OUTLINE OF THE DISSERTATION</td>
<td>51</td>
</tr>
<tr>
<td>CHAPTER 2: ACRYLONITRILE BUTADIENE STYRENE (ABS) AND POLYCARBONATE (PC) FILAMENTS THREE-DIMENSIONAL (3-D) PRINTER EMISSIONS-INDUCED CELL TOXICITY</td>
<td>55</td>
</tr>
<tr>
<td>2.1. INTRODUCTION</td>
<td>57</td>
</tr>
<tr>
<td>2.2. MATERIALS AND METHODS</td>
<td>58</td>
</tr>
<tr>
<td>2.2.1. Emissions collection setup</td>
<td>58</td>
</tr>
<tr>
<td>2.2.2. Emissions characterization</td>
<td>60</td>
</tr>
<tr>
<td>2.2.2.1. Particle size and concentration in cell culture medium</td>
<td>60</td>
</tr>
<tr>
<td>2.2.2.2. Morphology and elemental composition of particles in medium</td>
<td>61</td>
</tr>
<tr>
<td>2.2.2.3. Analysis of organic compounds collected in the cell culture medium</td>
<td>61</td>
</tr>
<tr>
<td>2.2.3. In vitro toxicity evaluation of collected 3-D printer emissions</td>
<td>62</td>
</tr>
<tr>
<td>2.2.3.1. Quantitation of endotoxin</td>
<td>62</td>
</tr>
<tr>
<td>2.2.3.2. Cell culture</td>
<td>62</td>
</tr>
<tr>
<td>2.2.3.3. Cell viability assay</td>
<td>62</td>
</tr>
<tr>
<td>2.2.3.4. Cell membrane damage</td>
<td>63</td>
</tr>
</tbody>
</table>
CHAPTER 3: PULMONARY AND SYSTEMIC TOXICITY IN RATS FOLLOWING INHALATION EXPOSURE OF 3-D PRINTER EMISSIONS FROM ACRYLONITRILE BUTADIENE STYRENE (ABS) FILAMENT

3.1. INTRODUCTION .................................................................................................................. 99
3.2. MATERIALS AND METHODS .......................................................................................... 101
  3.2.1. Three-dimensional printer emissions inhalation exposure system .................................. 101
  3.2.2. Three-dimensional printer settings for ABS filament .................................................... 102
  3.2.3. ABS emissions collection and characterization ........................................................... 104
    3.2.3.1. Particle collection and characterization ................................................................. 104
    3.2.3.2. VOCs collection and characterization .................................................................. 104
  3.2.4. Animals ..................................................................................................................... 105
  3.2.5. Experimental design .................................................................................................. 105
  3.2.6. Particle deposition estimates in the nasal passages, tracheobronchial, and alveolar regions ...... 106
    3.2.6.1. Particle deposition mass without clearance ......................................................... 106
    3.2.6.2. Particle deposition mass with clearance ............................................................. 106
  3.2.7. Bronchoalveolar lavage analysis ............................................................................... 107
    3.2.7.1. Bronchoalveolar lavage fluid collection and cytology ........................................ 107
    3.2.7.2. Transmission electron microscopy (TEM) staining of BAL cells ....................... 107
    3.2.7.3. Scanning electron microscopy (SEM) images of lungs ....................................... 108
    3.2.7.4. Total protein and LDH activity ............................................................................ 108
    3.2.7.5. Surfactant proteins A and D ............................................................................... 108
    3.2.7.6. BALF cytokines levels ......................................................................................... 108
  3.2.8. Oxidative stress markers ......................................................................................... 109
  3.2.9. Blood processing and analysis ................................................................................. 109
    3.2.9.1. Characterization of blood cells and hematological parameters ......................... 109
    3.2.9.2. Serum chemistry profile .................................................................................... 110
    3.2.9.3. Serum cytokine levels ....................................................................................... 110
    3.2.9.4. Serum immunoglobulin E .................................................................................. 110
  3.2.10. Lung and nasal passages histopathological evaluation ............................................ 110
  3.2.11. Statistical analysis .................................................................................................. 111
3.3. RESULTS ............................................................................................................................. 111
  3.3.1. ABS printing emissions characterization .................................................................... 111
    3.3.1.1. Particle mass and count concentrations ............................................................. 111
    3.3.1.2. Particle size ....................................................................................................... 113
### LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>three-dimensional cellular system, referring to the <em>in vitro</em> human bronchial epithelial airway cell model</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>ABS</td>
<td>acrylonitrile-butadiene-styrene</td>
</tr>
<tr>
<td>ACGIH®</td>
<td>American Conference of Governmental Industrial Hygienists</td>
</tr>
<tr>
<td>AET-I</td>
<td>alveolar epithelial type I cells</td>
</tr>
<tr>
<td>AET-II</td>
<td>alveolar epithelial type II cells</td>
</tr>
<tr>
<td>AgBB LCI</td>
<td>Committee for the Health Assessment of Building Products’s Lowest Concentration of Interest</td>
</tr>
<tr>
<td>ALB</td>
<td>albumin</td>
</tr>
<tr>
<td>ALI</td>
<td>air-liquid interface</td>
</tr>
<tr>
<td>ALKP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AM</td>
<td>additive manufacturing</td>
</tr>
<tr>
<td>AMF</td>
<td>additive manufacturing file</td>
</tr>
<tr>
<td>AMs</td>
<td>alveolar macrophages</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BALT</td>
<td>bronchus-associated lymphoid tissue</td>
</tr>
<tr>
<td>BJ</td>
<td>binder jetting</td>
</tr>
<tr>
<td>CAD</td>
<td>computer-aided design</td>
</tr>
<tr>
<td>CDPH SM</td>
<td>California Department of Public Health Standard Method</td>
</tr>
<tr>
<td>CREL</td>
<td>chronic reference exposure level</td>
</tr>
<tr>
<td>DED</td>
<td>directed energy deposition</td>
</tr>
<tr>
<td>DEHP</td>
<td>phthalate plasticizer</td>
</tr>
<tr>
<td>DLP</td>
<td>digital light processing</td>
</tr>
<tr>
<td>E∑VOC</td>
<td>sum of the ten highest detectable volatile organic compounds normalized by the mass of filament consumed</td>
</tr>
<tr>
<td>EBAM</td>
<td>electron beam AM</td>
</tr>
<tr>
<td>EBM</td>
<td>electron beam melting</td>
</tr>
<tr>
<td>EDS</td>
<td>energy dispersive spectroscopy</td>
</tr>
<tr>
<td>EDX</td>
<td>energy dispersive x-ray detector</td>
</tr>
<tr>
<td>EPA</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>ERs</td>
<td>emission rates</td>
</tr>
<tr>
<td>FDM™</td>
<td>fused deposition modeling</td>
</tr>
<tr>
<td>FE-SEM</td>
<td>field emission scanning electron microscope</td>
</tr>
<tr>
<td>FFF</td>
<td>fused filament fabrication</td>
</tr>
<tr>
<td>FMPS</td>
<td>fast mobility particle sizer</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GIT</td>
<td>gastro-intestinal-tract</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin (stain)</td>
</tr>
<tr>
<td>HAPs</td>
<td>hazardous air pollutants</td>
</tr>
<tr>
<td>HIPS</td>
<td>high impact polystyrene</td>
</tr>
<tr>
<td>IAQ</td>
<td>indoor air quality</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICRP</td>
<td>International Commission on Radiological Protection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10, anti-inflammatory cytokine</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>Interleukin-12p70 (IL-12)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Interleukin-17A</td>
</tr>
<tr>
<td>IM</td>
<td>interstitial macrophages</td>
</tr>
<tr>
<td>IVIVE</td>
<td>in vivo to in vitro dose extrapolations</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LOAEC</td>
<td>lowest observed adverse exposure concentrations</td>
</tr>
<tr>
<td>LOAELEs</td>
<td>lowest observed adverse effects levels</td>
</tr>
<tr>
<td>LOM</td>
<td>laminated object manufacturing</td>
</tr>
<tr>
<td>LT-MC</td>
<td>macrophage-mediated clearance</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>ME</td>
<td>material extrusion</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>macrophage inflammatory protein 1-alpha</td>
</tr>
<tr>
<td>MJ</td>
<td>material jetting</td>
</tr>
<tr>
<td>MJM</td>
<td>multi-jet modeling</td>
</tr>
<tr>
<td>MPPD</td>
<td>multiple-path particle deposition model</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Mucin 5AC, main mucin produced by the goblet cells</td>
</tr>
<tr>
<td>NHBE</td>
<td>human primary bronchial epithelial cells</td>
</tr>
<tr>
<td>NM</td>
<td>laminated object manufacturing</td>
</tr>
<tr>
<td>NOAEC</td>
<td>no observed adverse effect concentrations</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no observed adverse effect levels</td>
</tr>
<tr>
<td>NPs</td>
<td>nanoparticles</td>
</tr>
<tr>
<td>NPs</td>
<td>nanoparticles</td>
</tr>
<tr>
<td>NTA</td>
<td>nanoparticle tracking analysis</td>
</tr>
<tr>
<td>OBIV</td>
<td>in vivo organ burden data for in vitro dose setting</td>
</tr>
<tr>
<td>OS</td>
<td>oxidative stress</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid-Schiff</td>
</tr>
<tr>
<td>PBF</td>
<td>powder bed fusion</td>
</tr>
<tr>
<td>PC</td>
<td>polycarbonate</td>
</tr>
<tr>
<td>PET</td>
<td>polyethylene terephthalate</td>
</tr>
<tr>
<td>PETT</td>
<td>taulman t-glase</td>
</tr>
<tr>
<td>PLA</td>
<td>polylactic acid</td>
</tr>
<tr>
<td>PMNs</td>
<td>polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PNCs</td>
<td>particle number concentrations</td>
</tr>
<tr>
<td>PNERs</td>
<td>number emissions rates</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>REL</td>
<td>Recommended Exposure Limit</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SABM™</td>
<td>Small Airway Epithelial Cell Basal Medium</td>
</tr>
<tr>
<td>SAEC</td>
<td>small airways epithelial cells</td>
</tr>
<tr>
<td>SAGM™</td>
<td>Small Airway Epithelial Growth Medium</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SL</td>
<td>sheet lamination</td>
</tr>
<tr>
<td>SLA</td>
<td>stereolithography</td>
</tr>
<tr>
<td>SLS</td>
<td>selective laser sintering</td>
</tr>
<tr>
<td>SP-A</td>
<td>surfactant protein A</td>
</tr>
<tr>
<td>SP-D</td>
<td>surfactant protein D</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>STL</td>
<td>stereolithography</td>
</tr>
<tr>
<td>SVOC</td>
<td>semi-volatile organic compounds</td>
</tr>
<tr>
<td>TAC</td>
<td>Total Antioxidant Capacity</td>
</tr>
<tr>
<td>TB</td>
<td>tracheobronchial region</td>
</tr>
<tr>
<td>TEER</td>
<td>trans-epithelial electrical resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TLV®</td>
<td>Threshold Limit Value</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TPE</td>
<td>thermoplastic elastomer</td>
</tr>
<tr>
<td>TVOC ERs</td>
<td>total VOC emission rates</td>
</tr>
<tr>
<td>TVOCs</td>
<td>total VOCs</td>
</tr>
<tr>
<td>TVOC&lt;sub&gt;WL&lt;/sub&gt;</td>
<td>TVOC mass yield per unit mass of filament</td>
</tr>
<tr>
<td>TWA</td>
<td>time-weighted average</td>
</tr>
<tr>
<td>UFPs</td>
<td>ultrafine particles</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VOCs</td>
<td>volatile organic compounds</td>
</tr>
<tr>
<td>VP</td>
<td>Vat photopolymerization</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. ADDITIVE MANUFACTURING

Based on the approach by which a product is created, there are three types of manufacturing processes: subtractive, formative, and additive [1]. Additive manufacturing (AM) is a layer-based automated fabrication process of joining materials to make scaled 3-dimensional physical objects without using part-dependent tools [2, 3]. AM provides the next series of major expertise of the entire manufacturing technology due to flexibility in design, conception, and customization, which also explain the phenomenal expansion and evolution in recent years [4].

There are primarily eight process steps [5, 6] in AM:

- Step 1 – 3-dimensional (3-D) model creation (conceptualization): a 3-D computer-aided design (CAD) data set or model is created representing the part being produced using CAD design software or a 3-D object scanner or other imaging technologies such as computerized tomography scanning (CT-Scanning).
- Step 2 – STL file creation (tessellation): the 3-D data set is converted to an STL/AMF file format and sliced into digital layers using a computer and special software, resulting in a set of contoured virtual slices with even thickness.
- Step 3 – STL file transfer: the STL file is transferred to the AM machine, where it is manipulated to ensure the correct size, position, and orientation of virtual slices for building.
- Step 4 – Machine set up: consumables are loaded, and the AM machine is appropriately set up prior to the build process (build parameters like the material constraints, energy source, layer thickness, and timings).
- Step 5 – Building the part: this is an automated process; however, monitoring is needed to ensure no errors have taken place like running out of material, power, or software glitches.
- Step 6 – Removal of the build part: this part requires interaction with the machine as the part is removed from the build platform and its support structure.
- Step 7 – Post-processing: the part is cleaned up before use which requires careful, experienced manual manipulation.
• Step 8 – Part is ready to be used: parts may also require additional treatment before they are acceptable for use, such as priming and painting to give an acceptable surface texture and finish, or may also be required to be assembled together with other mechanical or electronic components to form a final model or product.

The major AM processes were classified in 2010, as per the American Society for Testing and Materials (ASTM) group “ASTM F42 – Additive Manufacturing”, into seven categories. The classification was done based on the type of materials used, the deposition technique, and how the material is fused or solidified. These categories are:

1) Vat Photopolymerization (VP)
2) Material Jetting (MJ)
3) Binder Jetting (BJ)
4) Material Extrusion (ME)
5) Powder Bed Fusion (PBF)
6) Sheet Lamination (SL)
7) Directed Energy Deposition (DED).

Table 1.1. includes details about these technologies, processes, the material used, a brief description, and advantages and disadvantages [7]. However, one should consider that AM is continuously expanding; therefore, new technologies could develop in the future along with a broader range of materials.

Some believe that we are experiencing a new industrial revolution due to the complexity of AM and its impact [8]. Additive manufacturing has been found to have many benefits [9-12], such as:

1) The direct translation of design to the component, with the capability of printing fully functional items without any assembly operations.
2) Generation of parts with greater customization with no additional tooling or manufacturing cost, thus reducing or even eliminating tool investment costs.
3) Functional design allows the manufacturing of complex geometrical parts with internal features, variable thicknesses, and irregular shapes.
<table>
<thead>
<tr>
<th>Process category</th>
<th>Example Technology</th>
<th>Materials (printed “ink”)</th>
<th>Brief description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Vat photopolymerization (VP) | Stereolithography (SLA) | Photopolymers Ceramics | Uses ultraviolet (UV) light to initiate a chain reaction on a layer of resin or monomer solution. The monomers are UV-active and instantly convert to polymer chains after activation by photoinitiators in resin. After polymerization, a pattern inside the resin layer is solidified in order to hold the subsequent layers. | • High build speed  
• High level of accuracy and good finish  
• Large build areas | • Limited to photopolymers  
• High cost for supplies and materials  
• Lengthy post-processing time and removal from resin  
• Often requires support structures and post-curing |
| | Digital Light Processing (DLP) | Polymers Ceramics | The material is pumped and deposited in the form of droplets via the injection nozzle onto the substrate. The droplets then form a continuous pattern which solidifies to sufficient strength in order to hold subsequent layers of printed materials. | • Multi-material printing  
• Multicolor  
• High accuracy of deposition of droplets  
• Low waste | • Support material is often required  
• Low-strength material |
| Material jetting (MJ) | Multi-Jet Modeling (MJM) | Polymers Ceramics Composites Wax Metals Biologicals | | | |
| Binder/Jetting (BJ) | Powder bed and inkjet head printing | Waxes Composites Polymers Ceramics Glass Metals Hybrid | The material is selectively joined together using a liquid binding agent (e.g., glue). Inks may also be deposited in order to impart color. Once a layer is formed, a new one is created by spreading powder over the top of the object, and the process is repeated until the object is formed. Unbound material is used to support the object being produced, thus reducing the need for support systems. | • Full-color object printing  
• Wide material selection  
• Faster than others  
• Allows for a large number of different binder-powder combinations and various mechanical properties | • Not always suitable for structural parts  
• High porosities on finished parts  
• Additional post-processing adds time to the overall process |
|---|---|---|---|---|
| Plaster-based 3-D printing | Thermoplastics Composites Biologics | A continuous filament\(^1\) of a thermoplastic polymer is heated at the nozzle to reach a semi-liquid state and then extruded on the platform or on top of previously printed layers.  
\(^1\) The feedstock can contain additives such as metals, flame retardants, nanomaterials, etc. | Widespread use  
Inexpensive  
Scalable  
Multi-material printing  
Can build fully functional parts | Vertical anisotropy  
Accuracy and speed are low when compared to other processes, and accuracy of the final model is limited to material nozzle thickness  
Limited part resolution  
Poor surface finish |
| Powder bed fusion (PBF) | Selective Laser Melting (SLM) | Thermoplastics Composites Metals Glass Ceramic | Thin layers of very fine powders, which are spread and closely packed on a platform, are fused together with a laser beam or a binder. Subsequent layers of powders are rolled on top of previous layers and fused together until the final 3D part is built. | • Relatively inexpensive  
• High accuracy and details  
• Fully dense parts  
• High specific strength, and stiffness  
• Large range of material options | • Relatively slow speed  
• Size limitations  
• High power required  
• Powder handling and recycling  
• Support and anchor structure  
• Finish is dependent on powder grain size |
| --- | --- | --- | --- | --- | --- |
| Sheet lamination (SL) | Electron Beam Melting (EBM) | Metals | Is based on layer-by-layer cutting and lamination of sheets or rolls of materials. Successive layers are cut precisely using a mechanical cutter or laser. They are then bonded together (form-then-bond) or vice versa (bond then-form). | • Fast  
• Low cost  
• Ease of material handling | • Finishes can vary depending on paper or plastic material but may require post-processing to achieve the desired effect  
• Limited material use |
Directed energy deposition (DED)

<table>
<thead>
<tr>
<th></th>
<th>Metals</th>
<th>Ceramics</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Beam AM (EBAM)</td>
<td>Uses a source of energy (laser or electron beam), which is directly focused on a small region of the substrate and is also used to melt a feedstock material (powder or wire) simultaneously. The melted material is then deposited and fused into the melted substrate and solidified after the movement of the laser beam.</td>
<td>• Ability to control the grain structure to a high degree, which lends the process to repair work of high quality • Functional parts</td>
<td>• Finishes can vary depending on paper or plastic material but may require post-processing to achieve the desired effect • Low shelf life • Limited material use • Expensive precursors</td>
</tr>
</tbody>
</table>

Table 1.1. Classification of seven additive manufacturing processes categorized by ASTM International, materials types, short description, advantages, and disadvantages. Adapted from [5-7].
4) Flexible and lightweight component manufacturing with hollow or lattice structures facilitating application for a specific function and specific characteristics, thus providing substantial benefits in various industrial sectors.

5) The ability of direct manufacturing of components to their final or near-final shape with minimal to no additional processing.

6) Potential to approach zero waste manufacturing by optimizing and maximizing material usage.

7) A significant reduction in overall product development, validation, and manufacturing time, leading to quicker market launch.

8) Smaller operational footprint towards manufacturing a large variety of parts.

9) On-demand manufacturing and flexibility in responding to the continuous changes in market demand and moving away from projection-based manufacturing.

10) Excellent scalability from the prototype to full-scale production.

11) Reduced environmental impact and increased sustainability (generation of less waste, reduce material consumption, and reduction in transportation in the supply chain).

1.1.1. **Fused filament fabrication**

Fused filament fabrication (FFF, also known as Filament Freeform Fabrication) is derived from Fused Deposition Modeling (FDM™ technology). It is an extrusion-based technology and one of the most popular additive manufacturing processes. FFF has applications in many types of manufacturing sectors, such as biomedical [13-21], pharmaceutical [22-28], aerospace [29-31], automobile [32-34], construction [35-39], electrical and electronics [40-43], food [44-48], textile [49-52], jewelry [53], toys [54-56], sports [57-59], and many others [60-66]. Also, 3-D printed devices such as oxygen valves, mask frames, ventilators, nasal swabs, and door openers have been designed and manufactured to fight against the spread of the Covid-19 virus and assist hospitalized patients [67].

FFF is conceptually the simplest, affordable, and most readily available AM technology that offers industries and consumers alike the possibility to build objects using a spool of filament-based material (thermoplastic). This makes FFF unique compared to other AM technologies. As the 3-D printer releases the thermoplastic filament, this is fed
into a heating chamber in the printer's extruder assembly, where it is heated above its glass transition temperature. Once it becomes more viscous, it can flow out the printer's head through a nozzle as the extruder assembly moves. The FFF 3-D printer extruder assembly can move horizontally and vertically (X,Y and Z axis), tracing a path programmed into the 3-D object file. The extruder assembly releases the molten plastic onto the print bed to create the physical object by adding thin layers, one on top of another. Once the filament cools, it hardens, and then the object can be released from the print bed. Prior to printing, slicing software defines how to break the 3-D digital model into layers that the printer can print, as described in steps 1 through 3 at the beginning of this section [5, 64, 67].

Efforts have been made on evaluating the effects of various parameters on the precise control of the extrusion process and the quality and performance of the printed parts. It had been determined that FFF is a complex process that is dependent on a significant number of parameters, such as machine parameters (printing speed, raster angle, melt flow rate through the nozzle, airgap, layer thickness, infill density, build orientation, and temperature) and material parameters (thermal and mechanical) [67-72]. More importantly, the composition of the toxic pollutants released during 3-D printing varies with different reaction temperatures, residence times, oxygen or additive contents, and, obviously, filament type [73], indicating that these parameters may influence potential health outcomes resulting from exposure. Therefore, a literature review of FFF 3-D printer emissions composition and characterization serves as the focus of section 1.4.

1.1.2. Thermoplastics used in fused filament fabrication

Thermoplastics, also called thermosoftening plastics, are plastic polymers that become soft and moldable when heated above a specific temperature called the glass transition temperature and solidify again as they cool, making them reusable nearly indefinitely [74]. Thermoplastics have the simplest molecular structure, consisting of chemically independent macromolecule chains that associate through intermolecular forces. This facilitates thermoplastics to be remolded as the intermolecular interactions increase upon cooling, restoring the bulk properties [75]. Above its glass transition
temperature and below its melting point, a thermoplastic can change its physical properties drastically without an associated phase change. Most thermoplastics are rubbery within this temperature range due to alternating rigid crystalline and elastic amorphous regions, approximating random coils [76]. For aesthetic purposes, pigments can be added to thermoplastics. Also, additives or fillers can be added to the thermoplastic to improve specific properties such as thermal or chemical stability and UV resistance [77].

Acrylonitrile-Butadiene-Styrene (ABS) and polycarbonate (PC) filaments are the most common filaments used in industrial settings and in offices, schools, universities, libraries, and homes [78]. Examples of other filaments popular on the market are nylon, high impact polystyrene (HIPS), polylactic acid (PLA), polyethylene terephthalate (PET), taulman T-glase (PETT), and thermoplastic elastomer (TPE) [79].

1.1.2.1. Manufacturing and uses of Acrylonitrile-Butadiene-Styrene

Acrylonitrile-butadiene-styrene (ABS) polymer is the largest volume engineering thermoplastic used in the US. It is produced by graft polymerization of acrylonitrile and styrene monomers onto an elastomer, such as polybutadiene or a butadiene copolymer [80]. This graft terpolymer's property advantages include excellent toughness, resistance, good dimensional stability, and processability [80].

ABS was introduced in the US in the late 1940s. In the 1950’s, the technique of grafting styrene and acrylonitrile onto polybutadiene rubber was perfected, thus introducing the acrylonitrile-butadiene-styrene copolymers in use today [81]. Acrylonitrile provides heat resistance, chemical resistance, and surface hardness. The butadiene supplies toughness and impact resistance, while the styrene component promotes processability, rigidity, and strength. ABS contains over 50% styrene and varying amounts of butadiene and acrylonitrile [80]. Two USA commercial copolymer samples examined were found to contain 30 and 50 mg/kg (ppm) residual, unreacted acrylonitrile monomer [81].

ABS is used in piping, automotive components (instrument panels, armrests, interior trim panels, seat belt retainers, glove compartment doors, lift gates, wheel covers,
grilles, headlight, mirror housings, and decorative trim), appliance components (door liners and food compartments of household refrigerators, fans and bases of blenders), components of business machines, telephones and electrical and electronic equipment, pipe fittings, recreational vehicle components (e.g., snowmobile components, canoes, and interiors of small and large boats) [82]. Other uses of ABS copolymers include packaging (margarine tubs), luggage and cases, toys and sporting goods, and furniture.

1.1.2.2. Manufacturing and uses of Polycarbonate

Polycarbonate (PC) is an amorphous engineering thermoplastic material. The term polycarbonate applies to several types of polymeric materials, which contain repeating carbonate linkages [78]. To increase melt strength for extrusion and blow molding applications, trifunctional monomers are added to the reaction process. PC is characterized by higher thermal stability, higher rigidity, and higher glass transitions compared to ABS [83].

The two chemical synthesis routes of PC are interfacial and melt reactions. Both interfacial and melt-reaction pathways for PC synthesis use Bisphenol A (BPA) as a monomer. PC is synthesized by reacting acetone and phenol via an acid-catalyzed reaction recrystallized to a high purity with a suitable solvent [84].

Since the discovery in the 1950s, the two-common types of polycarbonates are:
1) Aromatic polycarbonates (the most common type of polycarbonates), and
2) Aliphatic polycarbonates (prepared with similar synthesis methods as aromatic polycarbonates but based on aliphatic building block).

Aromatic polycarbonates are the largest portion of this class of materials. Most often, the generic term polycarbonate refers to BPA polycarbonate.

PC’s unique combination of transparency, rigidity, ductility, heat resistance, and processability made the polymer successful in many applications such as automotive (tail and headlights, signal light lenses and housings, runway markers, blow-molded spoilers, instrument panels, and seat backs), appliances (food processors, electrical kitchen components, power tool housings, refrigerator drawers, and vacuum cleaner components), electronic and business equipment (computer parts and peripherals, connectors, terminal blocks, and telecommunication components), medical products
(tubing connectors, dialysis components, and devices, blood oxygenators, filter housings, lenses, gamma sterilization appliances, and surgical staplers), safety and sports (sports helmets, recreational vehicle hoods, windshields, headlights, boat propellers, and sunglasses lenses) [78, 83].

1.2. FUSED FILAMENT FABRICATION EMISSIONS CHARACTERIZATION

Considerable evidence shows that the FFF process using thermoplastics as feedstock releases a significant number of nanoparticles (NPs) and various organic compounds [73, 85-103]. Concerning the health hazards, there is growing recognition that the biological activity and adverse health effects of toxicants depend on their physicochemical characteristics [104]. This represents an area of interest to both the public and manufacturers, toxicologists, regulatory decision-makers, and risk assessors [105]. Identifying the contributors to health risk among the complex physical-chemical components found in FFF 3-D printer emissions is an important matter, representing this section’s focus.

1.2.1. Nanoparticles

There is no clear understanding of the relationship between NPs toxicity and their physicochemical properties except for size. Oberdörster et al. [106] suggested that some other key characteristics that might be important to understand include agglomeration state, shape, chemical composition, surface area, surface chemistry, surface charge, and porosity.

A PubMed and Google scholar literature search was performed to include relevant publications from 2000 up to December 2020 that evaluated and characterized the FFF printer-emitted particulates. As a result, this section will review the following: 1) particle number emissions rates (PNERs) and particle number concentrations (PNCs), 2) the particle temporal pattern, 3) particle size distribution, 4) particle mass, 5) morphology, 6) particle agglomeration and volatility, and 7) elemental characteristics.

Published studies have found that the PNERs from 3-D printing are estimated to range from approximately $10^7$ to $10^{12}$ particles/min and that the printing materials and
printing conditions can influence the emission of particulate matter [91-93, 95, 101]. Across the literature, it is generally observed that the emissions characteristics vary significantly, and some filaments produce higher particle numbers than others. Azimi et al. [93] characterized the PNERs from a total of 16 unique combinations of five popular commercially available makes and models of desktop 3-D printers and nine filaments, including ABS, PLA, HIPS, semitransparent nylon, laybrick (an imitation brick material of unknown chemical composition), laywood (an imitation wood material of unknown chemical composition), transparent PC, a semitransparent nylon-based plasticized copolyamide thermoplastic elastomer (PCTPE), and a transparent polyester resin filament called T-Glase. It has been determined that ABS filament generates highest PNERs, particles/min median values ranging from $2 \times 10^{10}$ to $9 \times 10^{10}$, followed by PC filament ($4 \times 10^{10}$), PCTPE ($2 \times 10^{10}$), T-Glase ($5 \times 10^{9}$), HIPS ($4 \times 10^{9}$), nylon ($2 \times 10^{8}$), PLA ($10^{8}$), laywood ($8 \times 10^{7}$), and laybrick ($6 \times 10^{7}$).

Kwon et al. [88] classified the printer filaments as high, medium, and low emitters according to their PNERs. Under manufacturer-recommended and at consistent-temperature conditions, filaments with PNERs $>10^{11}$ particle/min were defined as high emitters, filaments with PNERs between $10^9$ and $<10^{11}$ particle/min were defined as medium emitters, and lastly, filaments with PNERs less than the medium emitters were considered low emitters. For instance, high emitters included HIPS (PNERs = $2.13 \times 10^{11} - 5.75 \times 10^{11}$ particle/min) and nylon (PNERs = $1.05 \times 10^{11} - 4.34 \times 10^{11}$ particle/min); medium emitters included ABS (PNERs = $6.91 \times 10^{9} - 5.56 \times 10^{10}$ particle/min), and low emitters included PVA (PNERs = $7.97 \times 10^{7} - 1.23 \times 10^{9}$ particle/min), laywood (PNERs = $6.83 \times 10^{7} - 7.71 \times 10^{8}$ particle/min), and PLA (PNERs = $1.98 \times 10^{7} - 7.92 \times 10^{8}$ particle/min).

The PNCs were reported to range from approximately $10^3$ to $10^5$ particles/cm$^3$ for room measurements [89-91, 101] and approximately $10^4$ to $10^6$ particles/cm$^3$ for chamber measurements [88, 90, 91, 93, 95, 96, 107]. PNCs over the printing time are direct measurements of particle counts per unit volume (cubic centimeter) and do not depend on the assumptions of potential particle losses to tubing (as a function of length, curves, and material - conductive vs. non-conductive tubing) in a system. Increases in PNCs
value can, in the same way as PNERs, indicate a greater potential for a particles' inhalation.

The size distribution and the temporal pattern of PNCs have raised concerns regarding NPs exposure during FFF 3-D printing [88-91, 94, 95, 97-99]. A typically large peak of particles in the range of 20 to 100 nm occurs during the initial heating and first layer printing, followed by an exponential decay of PNCs (Figure 1.1) [95]. It is hypothesized that it is due to the pre-heating of the filament in the 3-D printer nozzle and/or the printing of solid layers for the objects' solid exterior [94, 95, 108, 109]. The PNCs reached the maximum during the first four minutes when initial heating occurred (0–2 min), and when the solid layer was printed (2–4 min). For example, when comparing ABS, PLA, PVA, HIPS, PCABS, nylon, bronzePLA, and PET, the average initial peak concentration was between $1.1 \times 10^6$ particle/cm$^3$ (PLA & HIPS) and $4.8 \times 10^6$ particle/cm$^3$ (PVA) [95]. After the initial particle burst, a decline in the particle concentration is observed, indicating a lower emission rate than the ventilation rate when printing the 50% infilled density layer, compared with the solid exterior layers. Also, the decline in the particle concentration for each filament shows slightly different decay trends, which could be due to different particle emission rates for each filament (Figure 1.1). When the solid exterior is printed during the end of the printing (last two minutes), the particle counts increase again. Based on these findings, Floyd et al. [95] concluded that when printing the first and last layers (solid hard-shell), particle emission rates are higher compared with printing the middle layers (50% infilled density core).
Figure 1.1. Particle number concentration (PNCs, #/cm$^3$) in the sub-half-micron size range (16.8 – 532.8 nm) as a function of time (min) for ABS, PLA, PVA, HIPS, PCABS, nylon, bronzePLA, and PET filaments. Reproduced from Floyd, E.L., et al. [95], Fume emissions from a low-cost 3-D printer with various filaments. Journal of Occupational and Environmental Hygiene, 2017. 14(7): p. 523-533 with permission from the Taylor & Francis Group. https://doi.org/10.1080/15459624.2017.1302587.
To study the nanoparticle formation, Youn et al. [110] recorded real-time measurement of the PNCs as a function of particle diameter and time during printing with PLA (Figure 1.2). For a 3 h sampling time, they assigned five measurement periods or phases as such: (1) background, (2) phase 1: a single 3-D printer operation, (3) phase 2: two 3-D printer operation, (4) phase 3: five 3-D printer operation, and (5) background. To confirm that the nanoparticle formation was inhibited, a 10 min break was taken between the second and third phases. They measured the room's size-resolved PNCs representing the background reading, showing a unimodal distribution with a peak at 90 nm. Once they turned on the first 3-D printer, phase 1, a bimodal distribution was distinguished with peaks at 15 and 90 nm. At this phase, they observed the formation of <20 nm NPs, while the 70 - 100 nm NPs increased slightly. When a second printer was turned on, phase 2, the PNCs with sizes ranging from 10 and 230 nm increased. An interesting pattern was noticed for the PNCs of 70 - 100 nm particles: at the beginning of phase 2, it was the same as that observed in phase 1; however, it progressively increased as a function of time. This could be explained by the agglomeration of the 10 - 30 nm NPs emitted during both phase 1 and phase 2 and the continuous emission of the 70 - 100 nm large single NPs. This theory is supported when during the break time (between phases 1 and 2), the 10 - 30 nm particle number concentration decreased, but the 70 - 100 nm particles remained similar to that measured in phase 2 [111]. During phase 3, when five printers were operated simultaneously, the 10 - 30 nm PNCs dramatically increased initially with the highest concentration of all sampling periods. After phase 3, when the printer was off, nanoparticle sampling was conducted to obtain an after-background reading. At this phase, inhibition of 10 - 30 nm particle emissions was confirmed, whereas the 70 - 100 nm particles remained at similar number concentrations during the after-background measurement due to nanoparticle agglomeration. This suggests that caution should be used after the print run and not only when the printer is operating, considering the particles are <100 nm and potentially have higher toxicity due to high surface area compared to their larger counterparts [106].
Figure 1.2. TEM images of fume particles on scales of: (a) 100 nm, (b) 250 nm, and (c) 2 µm. Reproduced from Floyd, E.L., et al. [95], Fume emissions from a low-cost 3-D printer with various filaments. Journal of Occupational and Environmental Hygiene, 2017. 14(7): p. 523-533 with permission from the Taylor & Francis Group. https://doi.org/10.1080/15459624.2017.1302587.
Gu et al. [86] studied particle volatility by measuring particle number size distribution at room temperature using a Fast Mobility Particle Sizer (FMPS) and after the particles passed through a thermo-denuder with a second FMPS. The temperature in the thermo-denuder increased stepwise by 50 °C from room temperature to 300 °C. Overall, PNCs size decreased at increased thermo-denuder temperatures suggesting that the emitted particles are volatile and start to evaporate from 150 °C. Concerning particles emitted from 3-D printing with ABS, particle sizes and concentrations did not change very much at 100 °C; however, at 150 °C, the number of particles over 50 nm decreased, and at 200 °C, the particle mode around 30 nm decreased significantly. Particles with a diameter of around 10 nm showed little change, even at 300 °C. Similarly, for HIPS, the particle size and number concentrations gradually decreased with the increase of temperature: from 60 - 70 nm at 23 °C to 34 - 39 nm at 150 °C, 29 nm at 200 °C, and 11 nm at 250 °C and 300 °C. At 250 °C, almost all particles over 60 nm had disappeared. For PETG, PNCs decreased by 32%, 55%, and 73% at 150 °C, 200 °C, and 250 °C, respectively. The major particle mode shifted greatly, from 70 nm at room temperature to 34 nm at 150 °C, and 11 nm at 250 °C. The authors conclude that the 3-D printer emitted particles are composed of semi-volatile organic compounds (SVOC), though SVOC-involved nucleation and condensation, as observed in previous studies [91, 98, 110] and discussed in detail in the earlier paragraph. Additionally, using a volatility tandem differential mobility analyzer, Mendes et al. [96] evaluated the volatility of emitted nanoparticles and found that the emitted particles were composed of high and low volatility compounds, consistent with the presence of organic compounds in the printer aerosol emissions.

Traditionally, the mass dose is used to interpret the airborne fine particle concentration, as the mass concentration usually corresponds with the traditionally used occupational exposure levels. However, sampling of NP mass is a challenging task given their small size, heterogeneity, and unique chemical/physical properties. Up to date, mass concentration data of the particles released during 3-D printing is limited.

Chan et al. [112] measured total and respirable particulate concentrations in a room (2.4 × 2.4 × 2.4 m) where three 3-D printers were operating with PLA filament, and
the adjacent hallway (1.2 × 6.1 × 3.4 m) leading into the printing room. Sampling in the printing room was performed at a height simulating the seated position of a facility user. In the hallway just outside the printing room, air sampling was performed at the approximate breathing height of 1.2 m. During the sampling, the door connecting the printing room and hallway remained open because it is a usual practice at the facility. Two scenarios were considered: a “typical-case” when the three printers run sequentially for 155 min, and a “worst-case” scenario when all three printers run concurrently for 97 min. They found that the total particulate levels were higher in the worst-case scenario (0.7 mg/m$^3$) than the typical-case scenario (0.3 mg/m$^3$) in both settings. However, the printing room’s respirable particulate levels were lower in the worst-case scenario (0.4 mg/m$^3$) than the typical-case scenario (0.8 mg/m$^3$).

In another study, Väisänen et al. [113] measured occupational particle mass concentration generated during different AM processes: ME, VP, PBF, MJ, and MJM processes. Samples were collected from the height of the breathing zone from the beginning to the end of the manufacturing process, ranging from 70 to 150 min. The particle concentrations were lower than reported by Chan et al. [112]. Dust concentration generated during ME were ranging between 0.01 mg/m$^3$ and 0.03 mg/m$^3$ (0.01 mg/m$^3$ mean, and standard deviation, SD, of 0.01). They also noted that printer malfunction temporarily increased the particle concentrations by two orders of magnitude. To summarize their other findings from testing other AM processes: VP generated dust concentrations between 0.02 and 0.12 mg/m$^3$, PBF concentrations varied between 0.01 and 0.47 mg/m$^3$, MJ concentrations were similar to ME, from 0.01 to 0.03 mg/m$^3$, and during MJM the concentrations during manufacturing varied from 0.03 mg/m$^3$ to 0.07 mg/m$^3$.

Characterizing emissions from desktop 3-D printers in laboratories and classrooms on college campuses, McDonnell [114] found that half of all particles were <150 nm in diameter, and particle size distribution was skewed towards a smaller average diameter. PC (1.1 mg/m$^3$) generated the maximum particle mass concentration followed by ABS (0.2 - 0.6 mg/m$^3$), nylon (0.25 mg/m$^3$), and PLA (0.01 mg/m$^3$) lastly. Using real-time aerosol mass spectrometry, Katz et al. [87] determined fluctuations in particle size
distribution and mass concentration throughout the printing period in a small office space. Over the 3 hrs. print run, two distinct peaks were observed, one at 50 min and another peak between 120 - 150 min after the printer started. Steinle [90] reported that a 165 min print run in a small, unventilated room increased the ultrafine aerosol concentrations by 2,000 particles/cm\(^3\), and methyl methacrylate released during printing with PLA reached a peak of 21 µg/m\(^3\) and was still detectable even 20 hrs. after printing. Based on SMPS measurements inside a test chamber enclosure, Zontek et al. [115] found that the highest number and mass aerosol concentration of particles was generated during heating (80 µg/m\(^3\)) and printing (7.6 µg/m\(^3\)). Additionally, keeping the 3-D printer door closed reduced the number and mass concentration by 95%, thus proving the effectiveness of the enclosure by reducing the particle count in the breathing zone. Similarly, Dunn et al. [116] showed a 98% reduction in UFPs concentrations when an extruder head emission control was installed during printing in a test chamber. Furthermore, when evaluated the efficiency of this control in a simulated makerspace with 20 printers operating, it was found that with the engineering controls in place, the particle counts were reduced significantly to or at below background levels (< 1000 particles/cm\(^3\)) compared to the 20,000 particles/cm\(^3\) without the engineering controls in places. Thus, this study showed that including an engineering control to 3-D printers significantly reduces emissions to the work environment.

Overall, in these findings [112-114], the particle mass concentration was lower compared to American Conference of Governmental Industrial Hygienists’ (ACGIH\(^{®}\)) Threshold Limit Values (TLV\(^{®}\)) of 10 mg/m\(^3\) and 3 mg/m\(^3\), respectively [117]. There is a need for sampling equipment able to sample physical characteristics such as surface area, known to be a more appropriate metric for nanoparticle toxicity. Because NPs have such low mass, gravimetric analysis alone might be insufficient for the expression of exposure, and the number and/or surface area needs to be included to understand dose. Also, it is important to distinguish between background levels of NPs and incidental 3-D printer NPs. For example, Dunn et al. [118] reported free carbon nanotube (CNT) and carbon nanofiber (CNF) at a 3-D printing shop using filament printers with unfilled and CNT and/or CNF-infused polyetheletherketone (PEEK) filaments (these materials have
never been used in the facility) raising the possibility that these materials are being released from the matrix during 3-D printing.

The image analysis (Figure 1.2. and Figure 1.3) of emitted particle morphology confirms the presence of two forms of 3-D printer particles: primary sub-half-micron particles (<500 nm) and secondary super-half-micron particles (500 nm - 20 μm) [95, 110]. The majority of particles were agglomerated near-spherical NPs ranging from 10 - 20 nm size that tends to agglomerate to form larger particles approximately 100 nm in size. The median primary particle size was ranging between 70 and 100 nm. The deposition fraction and deposited areas in the human respiratory tract depend, in part, on the aerodynamic particle size; therefore, the primary particle size is critical [106]. Floyd et al. [95] reported the presence of micron-sized rod-shaped filament fragments. They believe that these particles could play an important role in scavenging smaller particles such as those in the sub-half-micron range. Additionally, the rod-shaped fragments can pose more pulmonary risks due to the ability to be trapped in the small airways. The 3-D printer particle morphology is also discussed in more detail above (page 9) in the context of the nanoparticle formation when Youn et al. [110] recorded real-time measurement of the PNCs as a function of particle diameter and time during printing with PLA (Figure 1.4).
Figure 1.3. 3-D printer nanoparticle morphologies. Reproduced from Youn, J.-S., et al. [110], Characteristics of nanoparticle formation and hazardous air pollutants emitted by 3D printer operations: from emission to inhalation. RSC Advances, 2019. 9(34): p. 19606-19612 with permission from the Royal Society of Chemistry. https://doi.org/10.1039/C9RA03248G.
Figure 1.4. Real-time measurement of the particle number concentrations as a function of particle diameter and time. Reproduced from Youn, J.-S., et al. [110], Characteristics of nanoparticle formation and hazardous air pollutants emitted by 3D printer operations: from emission to inhalation. RSC Advances, 2019. 9(34): p. 19606-19612 with permission from the Royal Society of Chemistry. https://doi.org/10.1039/C9RA03248G.
A variety of materials were detected from the elemental composition analysis of particles emitted during 3-D printing [90, 103, 110, 119]. For ABS, the following elements chromium (Cr, most abundant), nickel (Ni), silicon (Si), chlorine (Cl), calcium (Ca), magnesium (Mg), sodium (Na), aluminum (Al), and sulfur (S) were also reported [119]. For PLA, particles mostly contain Fe, Al, and copper (Cu) [90, 103, 119], and other studies also reported Si and potassium (K) [110]. It is believed that the presence of transition metals in 3-D printer emitted particles could be due to the use of pigments and additives added during filament manufacturing, residual catalysts from the polymerization process, the equipment used to extrude plastic resin into filament, and the FFF 3-D printer nozzle [119]. The transition metals could be linked to the reactive oxygen species (ROS) formation, which is the major mechanism of nanoparticle toxicity, leading to potential human health adverse effects.

Stephen et al. [101] compared the PNERs from the 3-D printer to ultrafine particle (UFP) emission reported from cooking activities. They estimated that printing with PLA has a similar UFP emission rate as cooking with an electric frying pan, and printing with ABS has a similar emission rate estimate as grilling food on stoves at low power, but approximately an order of magnitude lower than gas or electric stoves operating at high power. The same study concluded that because the PNERs were greater than $10^{10}$ particles/min, 3-D printer emissions can be classified as “high emitters” according to criteria set by He et al. [120]. Schripp et al. [121] found that the FFF 3-D printer particle profiles (particle concentration and size distribution) were in the same range as laser-jet printers.

1.2.2. Volatile organic compounds

To fully evaluate the potential health concerns from FFF 3-D printing, an assessment of the complex mixture of gases released during printing that could deteriorate indoor air quality (IAQ) is needed. Previous studies have found that gas-phase emissions from 3-D printers may include odorants, irritants, and carcinogens [90, 93, 95, 119]. Commonly detected individual volatile organic compounds (VOCs) from 3-D printing included styrene [73, 90, 93, 95, 119], lactide [93, 95], caprolactam [93, 95], ethylbenzene [90, 93, 95, 119], xylenes [93, 95, 119], and aldehydes (acetaldehyde, formaldehyde) [95,
Monomers or derivatives of filament material were the speciated VOCs commonly detected [90, 93, 95].

A PubMed and Google scholar literature search was performed to include relevant publications from 2000 up to December 2020 that evaluated and characterized the volatile organic compounds released during FFF 3-D printing. As a result, this section will review the following: 1) the total VOC emission rates (TVOC ERs), 2) the specific VOCs released during the printing process, and 3) the relationship between VOCs and particle emissions.

Filament material type impacts the differences in the TVOC ERs and types of VOCs emitted. Within the same filament, the printer make and model influence the differences in the overall mass of the VOCs emitted [93]. For instance, ABS was found to release more TVOC than PLA [91, 93, 95, 119, 122]. However, other studies reported that TVOC ERs were higher for PLA than ABS [90]. The contrasting findings could be explained on the method employed by each researcher, supporting the need to use a defined test method so that comparative data can be obtained.

Azimi et al. [93] characterized the VOCs emissions from 16 unique combinations of five popular commercially available makes and models of desktop 3-D printers and nine filaments. The 3-D printer filaments were categorized in low emitters and high emitters, respectively, based on the sum of the ten highest detectable VOCs normalized by the mass of filament consumed (E∑VOC). The high emitters, with E∑VOC > 40 μg/min, were nylon, followed by semitransparent nylon-based plasticized copolyamide thermoplastic elastomer (PCTPE), ABS, laybrick (an imitation brick material of unknown chemical composition), and laywood (an imitation wood material of unknown chemical composition). The low emitters, with E∑VOC < 40 μg/min, were PLA, transparent PC, and a transparent polyester resin filament called T-Glase.

To measure the 3-D printer TVOCs, the above-presented studies used the two most common methods, based on photoionization detection and GC/MS principle, respectively. On the other hand, Ding et al. [85] determined the TVOC mass yield per unit mass of filaments (TVOCWL) via the total weight loss of organic compounds that were released during printing per unit mass of filaments after extrusion (μg/g or %). It was noted
that TVOCWL was 0.03% (0.29 mg/g) for PLA, 0.21% (2.14 mg/g) for ABS, and 2.14% (21.38 mg/g) for PVA. Similar to previous findings [93, 95, 119], which used the semi-quantitative methods mentioned above, the levels of TVOCWL were larger for ABS compared to PLA. Furthermore, to support the emissions variance between the materials, the relative stabilities were calculated based on the difference between the pyrolysis and nozzle temperature. The relative stability of PVA was lower (45 °C - 150 °C) in comparison to ABS (110 °C - 196 °C) and PLA2 (120 °C - 173 °C), which was consistent with the UFP emissions pattern [85].

To investigate the effects of printer brand on VOCs emissions, two similar filaments (ABS and PLA) were compared when using two different printers (printer A and E) [122]. It was has established that the printer brand had a significant effect on emissions yields and TVOC ERs. For ABS, printer E emitted twice as much TVOCs and styrene as printer A, while for PLA, the reverse was noticed. The particle numbers were influenced similarly by the difference in printer brands.

Stefaniak et al. [119] evaluated the effect of color on TVOC ERs. It was found that when printing with different filament types with the same color, the TVOC ERs were significantly different. For example, ERs for blue ABS (2385 ± 82 μg/h) were higher compared to transparent blue PLA (131 ± 37 μg/h) and below the limit of detection for ocean blue PLA. Also, red ABS (2383 ± 357 μg/h) was significantly higher than true red PLA (49 μg/h). Additionally, the color effect on the TVOC ERs within a given type of filament varied. For ABS, the ERs for natural color were significantly higher than black. At the same time, for PLA, there were no statistical differences among PLA filament colors (ocean blue, transparent blue, true red, and army green) [119]. Similarly, for ABS, the ERs decreased from red > green > white, while for PLA, there was no difference between white, black, and natural filament [122].

The impact of nozzle and printer bed temperatures on TVOC ERs was also explored [93, 119, 122], and conflicting data were discovered. While some studies [122] suggest that an increase in nozzle temperature has a significant effect on the VOC and particle emission, other studies found no clear relationship between TVOC ERs and either bed or nozzle temperatures [93]. However, the PNERS are greatly affected by the nozzle
temperature [119, 123], with ERs 100 - 10,000 times higher than the ERs at the lowest temperature. Moreover, Davis et al. [122] suggest that an increase in nozzle temperature has a greater impact on PNERs (increases by about 128%) and particle mass emissions (increases by about 106%) than it does for VOC emissions.

The individual VOC information is more critical than the total TVOC ERs since VOCs' toxicity varies for each chemical. As already mentioned, the VOC emission types vary upon the filament used, therefore, depending on how the filament is formulated. It was found [122] that between ABS, PLA, PVA, and HIPS filaments, ABS had the most compounds detected with 177 individual VOCs, followed by 70 compounds from HIPS, 57 compounds from PLA, 49 compounds from PVA, and 47 compounds from nylon. As anticipated, styrene and its derivatives, nitriles, and benzothiazole (chemicals with nitrogen like acrylonitrile and ethylbenzene used in the production of styrene), were emitted from ABS and HIPS filaments [93, 95, 119, 122]. The primary individual VOC emitted from nylon, PCTPE, laybrick, laywood, polycarbonate, and T-Glase filaments was caprolactam [93, 122]. Lactide was found as the primary individual VOC emitted from PLA filaments [93].

A study to evaluate correlations between VOCs and particles emitted during printing was conducted [122]. For ABS filament, an increase in styrene and benzothiazole emission rates (ERs) was strongly associated with increased particle mass ER and PNERs. For HIPS, because styrene is the main compound released during printing (similar to ABS), the data point for PNERs versus styrene or TVOCs falls in the range of ABS data points, however, not for the particle mass emission. HIPS total particle mass was much higher than that of ABS, also due to larger particle sizes. For PLA, a weak positive relationship was observed between lactide and total particle mass or PNERs, but a strong positive relationship was observed between formaldehyde ER and particle mass ER. Interestingly, lactide's ER from a PLA with additives (Special PLA) was within the range of other PLAs tested; however, it emitted more particles (mass and number), as well as greater TVOC ER. This may indicate that this Special PLA's emissions were associated with its additives rather than the bulk material. Emissions from PVA behaved similarly to PLA and lower-emitting ABS for both gas and particle emissions. For Nylon
filament, an inverse relationship between caprolactam emissions and particle emissions was noted, where the higher VOC emitting nylon filament emitted fewer particles [122].

New studies indicate that ozone reacts with laser printer-generated VOCs to form secondary organic aerosols [124, 125]. To test this hypothesis in regard to 3-D printing, Stefaniak et al. [119] measured the ozone emissions rates and identified the carbonyl compounds from ABS and PLA emissions. Indeed, compounds such as 4-oxopentanal, glyoxal, methylglyoxal, and benzaldehyde were identified in both filament emissions [119], confirming ozone-initiated reactions of alkene compounds from the 3-D printers’ emissions. Therefore, it is essential to identify these compounds as well, besides the particles and VOCs, to understand the complete array of potential factors that could cause respiratory or other health effects upon exposure to the 3-D printer emissions.

Studies have compared the 3-D printer TVOC ERs by filament material to those from laser printers, copiers, and computers [119, 122]. On average, the TVOC ERs were lower than those from laser printers and dry process copiers. However, some of the high emitting filaments like ABS and nylon had similar TVOC ERs as lower-emitting laser printers and personal computers. However, care should be taken considering that when filaments include additives like bronze and nanotubes, VOC emissions may increase [95] or decrease [73].

In conclusion, FFF printer emissions consist of complex mixtures, and several reasons for the observed differences in emissions identified among studies, include variables such as the printer brand, composition of the polymer filament, printer extrusion temperatures, and sampling methods used by investigators. Therefore, it is critical to characterize 3-D printing emissions consistently and accurately. This can be done using a defined test method to obtain comparative data. The experimental setup (i.e., test environment and exposure methods) and analysis techniques varied among studies [73, 90, 91, 93, 95, 119], making it difficult to compare data between studies or with other emissions resources.
1.3. HEALTH AND SAFETY CONSIDERATION OF FUSED FILAMENT FABRICATION 3-D PRINTER EMISSIONS

As already mentioned in the previous section, during the thermal decomposition of filaments, hazardous pollutants such as NPs and VOCs are released into the air [90, 93, 101, 108, 119, 122, 126]. In this section, potential toxicological effects from exposure to these two components and possible mechanisms of toxicity will be reviewed.

1.3.1. Nanoparticles

1.3.1.1. Deposition behavior of nanoparticles emitted from 3-D printers

As with any emerging technology, the knowledge and research about health and safety risks from exposure to FFF 3-D printer emissions is unclear and understudied. Concerns have been raised regarding FFF 3-D printing, as the printer-emitted particles are smaller than 100 nm, with the potential to penetrate deeper into the lower respiratory tract upon inhalation, and infiltrating the extra-thoracic and conducting systems [127]. Although it is not the focus of our study, it is worth mentioning that the surface contaminants of emitted particles can leach out of the parts [128] and potentially result in enhanced toxicity.

Understanding the deposition behavior of NPs emitted from 3-D printers can help estimate their potential toxicity in terms of human health perspective. Youn et al. [110], using multiple-path particle dosimetry (MPPD) model, which evaluated deposition of 10 nm to 20 mm particles over the entire respiratory system, translated exposure to 3-D printed NPs to inhaled deposited dose by determining the number of NPs that could be deposited in the human respiratory tract as a function of airway generation number (Figure 1.5). Based on the total particle number dose (#) per human airway generation number, approximately 70% of the NPs are deposited in the human respiratory system ranging in generation number from 16 to 28, with the maximum particle dose occurring (4 × 10^6 particles) at the 20th airway generation (Figure 1.5(a)), implying that the NPs deposited in the pulmonary region which consists primarily of the alveolar region (AL) [129]. Furthermore, examining the particle number dose as a function of both particle size and airway generation number (Figure 1.5(b)), they estimated that the highest particle
number dose ($6.6 \times 10^6$ particles) was observed at the nanoparticle size of 15.1 nm and the 19th airway generation. Overall, the 10-40 nm NPs showed the highest particle number dose range, located between the airway generation number 16 and 22, indicating that a large number of NPs were deposited in the lower respiratory tract compared to that of the upper respiratory tract [110]. Moreover, when evaluating the total particle deposition density (#/cm$^2$) per human air generation number, the authors found that approximately 60% of the deposition density appeared in the airway number between 1 and 5, at the starting point of the tracheobronchial region (TB) in the upper respiratory tract, with the maximum deposition density occurring in the 3rd airways ($3.7 \times 10^4$ #/cm$^2$) (Figure 1.6(a)). This is likely because the surface areas of the airway generation number from 1-5 are approximately 2-530 times smaller than that of the surface areas of the airway generation ranging from 6-28 [130]. Overall, based on both nanoparticle diameter and airway generation number, the highest deposition densities were observed for particles with diameters between 10-40 nm and were higher in the upper respiratory system due to a smaller surface area (Figure 1.6(b)).
Figure 1.5. (a) Total particle number dose (#) per human air generation number and (b) particle number doses per human airway generation number and nanoparticle diameter. Reproduced from Youn, J.-S., et al. [110], Characteristics of nanoparticle formation and hazardous air pollutants emitted by 3D printer operations: from emission to inhalation. RSC Advances, 2019. 9(34): p. 19606-19612. 
https://doi.org/10.1039/C9RA03248G with permission from the Royal Society of Chemistry.
Figure 1.6. (a) Total particle deposition density (# cm$^2$) per human air generation number and (b) particle deposition density per human airway generation number and nanoparticle diameter. Reproduced from Youn, J.-S. [110], et al., Characteristics of nanoparticle formation and hazardous air pollutants emitted by 3D printer operations: from emission to inhalation. RSC Advances, 2019. 9(34): p. 19606-19612. https://doi.org/10.1039/C9RA03248G with permission from the Royal Society of Chemistry.
Using a different approach, Park et al. [131] used the International Commission on Radiological Protection (ICRP) lung deposition model to estimate inhalation exposure doses for a reference human during 3-D printing. They estimated that more than 95% (1.3 x 10⁹ nano-sized particles/kgbw/g) of the total inhaled dose on a particle number basis were inhaled when high emitters filaments (HIPS and nylon) were used for printing and 63%-72% (6.0 x 10⁶ nano-sized particles/kgbw/g) when low emitters (PLA, PVA, or Laywood) were used, respectively. For the particles larger than 1.0 μm, less than 0.1% of emissions represented the inhaled dose. However, the mass of particles between 1.0 and 10 μm accounted for a larger proportion of the total inhaled dose (about 60%-90%). Among the filament’s types, the high emitters produced an inhalation dose of about 82%-91% of the total inhaled dose (150-230 ng/kgbw/g) compared to the low emitters 62%-76% (10-30 ng/kgbw/g). The deposited fractions in the head airway (HA), the TB, and the AL were further calculated. It was established that 63.6% (3.1 x 10⁸ particles/kgbw/g) from the total inhaled dose, deposited in the respiratory regions, with the AL accounting for the largest proportion (23%-44%) of the total inhaled dose. Specifically, about 40% of the inhaled dose, was released from the high emitters (5.8 x 10⁶ particles/kgbw/g) and about 27% (2.0 x 10⁶ particles/kgbw/g) for the low emitters, respectively. Additionally, it was found that a smaller proportion of emitted particles (16%-41%) deposited in the respiratory regions on a mass basis than on a particle number basis. For instance, from 5.0-20.2 ng/kgbw/g to 27.7-43.3 ng/kgbw/g for the low emitters and the high emitters filaments. Despite the large, inhaled dose on a mass basis from the high emitters (170-280 ng/kgbw/g), only 17% was deposited, whereas the low emitters on a particle number basis was 58.7%-70.3%. This could be explained by the larger particle size released by the low emitters than the high emitters. These larger particles had a greater effect on the total mass of the deposited dose. Interestingly, it was estimated that 3-D printers release a higher number concentration of inhaled particles compared to consumer products such as nano-enabled consumer spray products [132].

Using MPPD and ICRP66 models, Byrley et al. [133] modeled inhaled deposited mass in different regions of the respiratory tract for different age groups (3 months, 23 months, 3 years, 9 years, 14 years, 18 years, and adults) and both sexes. The highest predicted deposition mass per surface area in the pulmonary region was for age groups...
3 months through 9 years, whereas deposited mass alone (not normalized) in the pulmonary region was predicted as highest in the 9-year-old through 18-year-old age groups. It was predicted that the overall deposition to be lower in females than in males, possibly due to the smaller tidal volume in females. However, the mass deposited per pulmonary surface area in the female model was slightly higher than in the male model. Clearance modeling predicted that most particles (84.9%) deposited in the pulmonary region were retained after 30 days from a single 2-hr exposure. A simulation of four weeks daily exposure with 2 hrs. of 3-D printer use every day predicted 67.9% retained mass in the pulmonary region in male adults at five-month post-exposure period, raising concerns about cumulative deposition for frequent 3-D printer users.

1.3.1.2. Mechanisms of nanoparticle induced-toxicity

Exposure to NPs poses a cause for concern as toxicity studies have shown that pulmonary exposure to NPs produces a greater adverse inflammatory response than larger particles of identical composition at equivalent mass concentrations [134]. The enhanced toxicity of nano-sized materials compared to the respective bulk materials arises from the fact that decreasing the size of the materials leads to an exponential increase in surface area relative to volume, thereby making them highly reactive [135, 136].

Next, three main possible mechanisms underlying NPs-mediated toxicity will be discussed: oxidative stress (OS), inflammation, and particle translocation. The molecular mechanism of NPs-induced toxicity is not fully understood, as OS and inflammation are interconnected [137].

1.3.1.2.1. Oxidative stress: concept and biomarkers

OS is one of the central mechanisms underlying the toxicity of NPs. OS results from the imbalance of reactive oxygen species (ROS) generation and antioxidant enzymes [138-141], and it plays a critical role in many diseases, such as pulmonary diseases, cancer, and arteriosclerosis [142-151]. Particle size, along with shape and surface area, play a decisive role in toxicity as the smaller the size, the more capable it is of triggering ROS formation due to the higher number of reactive sites on its surface [152-
It has been also noted that NP-induced biochemical catalysis is promoted by significantly accelerated chemical reactions with NPs attributed to the dangling bonds (immobilized free radicals) of the atoms located on the NP surface [155]. These free radicals affect biological systems through the oxidation of proteins, lipids, and nucleic acids. Therefore, the degree of OS can be measured indirectly by measuring the levels of protein oxidation/nitration, lipid peroxidation, and DNA/RNA damage.

When a cell suffers OS, antioxidants present in the cell respond to reduce and maintain the basal ROS generation. Total antioxidant capacity provides an overall measure for the cumulative effect of antioxidant defense capacity, including enzymatic and nonenzymatic systems [156, 157]. Hence, total antioxidant capacity has been considered a useful indicator of a system’s total antioxidant status, considering that various antioxidant enzymes, antioxidant vitamins, and micronutrients work in concert and interact with each other to maintain cellular ROS balance [158, 159]. Previous in vivo and in vitro studies reported a reduction in the antioxidant capacity following exposure to air pollutants [160], and metal oxide NPs such as MgO [161], TiO$_2$ and ZnO [162], selenium (Se) NPs [163], indicating a reduction in antioxidant defense mechanisms.

Phase I and II metabolic enzymes regulate the balance between the overproduction and destruction of ROS within the cell [164]. Glutathione peroxidase (GPx) is a major front-line defense phase II enzyme (selenoenzyme antioxidant enzyme), responsible for breaking down hydrogen peroxide and additional peroxides into less toxic compounds and reduces the formation of free hydroxyl radicals [165, 166]. GPx has been considered one of the most important antioxidant enzymes due to its higher affinity than catalase to hydrogen peroxide, a central redox signaling molecule and contributor to OS [167]. Animal studies have shown that neurons in mice lacking GPx were more susceptible to hydrogen peroxide-mediated toxicity than the wild-type mice [168], reinforcing that GPx is needed to deal with OS, and to eliminate ROS when exposed to toxicants. Moreover, it has been shown that deletion of the genes regulating glutathione-S-transferase activity, a large family of Phase II enzymes, influenced the level of DNA adducts and the development of lung cancer [169].
Protein carbonyl is a major marker of oxidative modification of proteins. Protein carbonylation results in irreversible protein dysfunction generated via metal-catalyzed oxidation or from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues [170, 171]. Introduction of carbonyl groups such as aldehyde, ketone, and lactam into the proline, arginine, lysine, and threonine amino acid side chains of proteins, and subsequent accumulation of protein carbonyl have been found after exposure to NPs [172, 173], indicating cellular injury and suggesting their potential role in disease pathogenesis [174]. The use of protein carbonyl as a potential marker for assessing OS is advantageous compared to the measurement of other oxidation products because of the relative early formation and carbonylated proteins' relative stability [175, 176].

Lipids are susceptible targets of oxidation because of their molecular structure that contains abundant reactive double bonds [177]. Lipid peroxidation is a series of reactions in cell membranes that produces lipid hydroperoxides as the main product, and many different aldehydes such as malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal formed as secondary products. The reaction is initiated by the formation of a carbon-centered radical within a polyunsaturated fatty acid of a complex lipid [178]. Malondialdehyde (MDA) is generated via peroxidation of polyunsaturated fatty acids (omega-3 and omega-6 fatty acids) and is one of the most well-studied markers of lipid peroxidation [179]. Increased MDA levels following pulmonary exposure to various NPs have been reported in the literature [173, 180-182].

1.3.1.2.2. Inflammation: concept and biomarkers

The second major mechanism concerning NPs-induced toxicity is inflammation. Recent research suggests that in the lung, particles (or soluble particle components) interact with the epithelial cells and resident macrophages (primary targets of inhaled toxicants). This result in activation of intracellular signaling cascades, triggering transcriptional activation of pro-inflammatory genes and onset of inflammatory responses [183]. These initial physio-pathological responses are crucial in modulating the onset and regulation of innate and adaptive immune responses, since excess or persistence of inflammation may induce or exacerbate respiratory, cardiovascular diseases, cancer, etc.
Molecular triggering mechanisms involved in prompting inflammatory reactions have been categorized into ROS’s direct formation with subsequent oxidative stress, interaction with the lipid layer of cellular membranes and its receptors and ion channels, and direct interactions with intracellular molecular targets [183]. Phagocytes, specifically alveolar macrophages (AMs) and polymorphonuclear neutrophils (PMN), and pro-inflammatory cytokines are known as significant components of inflammatory and immunologic reactions and applicable tools in evaluating NPs lung defense and injury [192, 193].

Airway macrophages are known as “the guardians of tissue repair in the lung.” [194] as their primary role is to keep the air spaces clear by removal of all foreign materials. AMs are long-lived cells derived from embryonic progenitors that colonize the airways with the first breath of a newborn [195] and self-renew under homeostatic conditions, representing the most abundant innate immune cells in the lung [196]. They are the first to encounter incoming toxicants and pathogens and help orchestrate the initiation and resolution of the lung's innate and adaptive immune response. To maintain lung homeostasis, macrophage populations within the lungs identify NPs as foreign bodies due to surface opsonization and take them up by endocytosis or phagocytosis, leading to coordinated induction of gene expression, antigen presentation, and cytokine production [197]. In response to lung injury, under an inflammatory state, resident AMs are supplemented by newly recruited monocyte-derived macrophages. These are called infiltrating AMs and are recruited from the bone marrow [198]; however, they remain short-lived after injury or sometimes last for several months after the disease resolves. As such, a rise in AMs in bronchoalveolar lavage fluid (BALF) has been known to occur in response to NPs exposure [199, 200].

AMs are critical effectors in the mechanism underlying the self-clearance of the pulmonary region. In alveoli, macrophages remove particles by phagocytosis and migrate towards the mucociliary escalator to be eliminated; this whole process is lasting several days. However, macrophages are less efficient in the phagocytosis of NMs than microparticles, making NMs staying longer in the alveolar space, increasing their probability of interacting and entering the alveolar epithelium and/or crossing the air-blood
barrier [201]. The recruitment of PMNs in response to the damaging effect of toxicants on macrophages plays an essential, enhanced role in this process. This neutrophil recruitment depends on the amount of AMs breakdown products being generated. Thus, more breakdown products will result in higher and more severe neutrophil influx, amplifying the effects of particles on the macrophage [202, 203]. That is why neutrophil ratio to macrophage counts in the BALF is often used as an indirect indicator to establish different particles’ toxicity [204, 205].

In response to injury, when the mucociliary clearance and the AMs are overwhelmed, the PMNs migrate from the pulmonary vasculature to the inflammatory site where they will produce several pro-inflammatory mediators, including chemokines that first attract other PMNs followed by other cell types like monocytes-macrophages and lymphocytes [206, 207]. However, in addition to their defensive role, PMNs are an essential source of cytotoxic and proteolytic products such as IL-1α, IL-8, IL-12, TNF-α, TGF-β, and GRO-α cytokines, which are implicated in tissue damage and inflammation [207]. Therefore, an increase of PMNs in the respiratory tract can be associated with significant damage and irreversible architectural changes in the lung [208-211].

Cytokines are small, non-structural proteins with low molecular weights that are produced by various types of cells, such as neutrophils, eosinophils, basophils, monocytes, and lymphocytes, that play a pivotal role in homeostasis by modulating and regulating immunity and inflammation responses [212]. Cytokine functions are pleiotropic and redundant, in that they accomplish multiple biological functions and often overlap, acting synergistically or antagonizing each other, and exhibit both negative and positive regulatory effects on various target cells. This is why cytokine interactions are often referred to as a network [193].

Because the cytokines are produced very early in the immune response to a stimulus, they are often measured to predict nanomaterials' immunomodulatory effects and the possibility of inflammation-mediated toxicity. About 200 cytokines are recognized and categorized based on which cell type they are produced from: either Th1 cells or Th2 cells. Lately, a third subset of Th cells (Th17) and T regulatory cells (Treg) are described, which show different cytokine profiles from Th1 and Th2 cells [193]. Macrophages
produce a plethora of cytokines, including TNF, IL-1, IL-6, IL-12, IL-15, IL-18, chemokines such as IL-8, MIP1, MIP-1, MCP-1, interferons, among others. Inflammatory cytokines are further divided into those involved in acute inflammation and those responsible for chronic inflammation [213]. The key cytokines mediating acute inflammatory reactions are IL-1, TNF-α, IL-6, IL-11, IL-8, and chemokines including G-CSF, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Of these, IL-1 (α and β), IL-6, and TNF are extremely potent inflammatory molecules. IL-1, IL-6, and TNF-α increase vascular permeability and, thus, cause swelling and redness associated with inflammation. IL-1 and IL-6 are responsible for fever reactions, whereas TNF-α stimulates endothelial cells and is responsible for hypotension. IL-8 is a chemokine that plays a vital role in the activation of neutrophils and their recruitment to the site of inflammation. Many cytokines act together to initiate and regulate the inflammation process. For example, IFN-γ plays a significant role in the inflammatory process. It can attract macrophages to sites where antigens are present [214]. During chronic inflammation, cytokine interactions result in monocyte chemotaxis to the inflammation site where IFN-γ, MCP-1, and other molecules activate the macrophages, while GM-CSF and TNF-α retain them at the inflammatory site [215, 216].

1.3.1.2.3. Translocation: concept and biomarkers of systemic toxicity

While the respiratory tract is considered the primary target organ for inhaled particles, research has demonstrated that extra-pulmonary organs such as aorta, heart, liver, spleen, and lymph nodes are also affected [217-220]. Adverse systemic effects induced by inhalation of NPs are suggested to result via two pathways. 1) the inflammatory or pro-thrombotic mediators can "spill-over" [221] into the interstitial space gaining access to the blood. These then get broadcast into the entire systemic circulation, reaching the secondary organs resulting in disorders. 2) particles' pass directly into the systemic circulation via uptake and translocation [222]. Once NPs enter the systemic circulation, they may accumulate in secondary organs and tissues. Uptake and translocation are believed to occur by two pathways: 1) NP translocation across the air-blood-barrier or 2) NP translocation across the gastro-intestinal-tract (GIT) walls. NPs which were eliminated from the lungs by mucociliary clearance towards the larynx and
swallowed can enter into the GIT and gain access to extrapulmonary organ either directly via blood circulation or via the thoracic lymph duct. Indeed, it has been already reported that nose-only inhalation exposure of rats to the ABS emission for 3 h induced significantly higher mean arterial pressure concomitant with the elevated resting arteriolar tone and impaired endothelium-dependent arteriolar dilation [223].

The exact mechanism of NP’s translocation is not clear yet. Thorley et al. [222] proposed that the NPs uptake and translocation across the pulmonary epithelium is controlled by alveolar type I epithelial cells but not by either the alveolar type II epithelial cells or by paracellular transport since the NPs were unable to penetrate the tight junctions. Riediker et al. [224] suggested the idea of the long-term, macrophage-mediated clearance (LT-MC) mechanism. This study emphasized the role of interstitial macrophages (IM) population in phagocytizing NPs and their long-term retention in the septal interstitial spaces. IM facilitate gradual re-entrainment of NPs on top of the epithelium for subsequent LT-MC towards the larynx and into the GIT. Re-entrainment can also occur across the alveolar epithelium and/or via interstitial-lymphatic clearance to bronchus-associated lymphoid tissue entering the epithelial surface at the airway epithelium for clearance by surface macrophages. Next, NP’s could move through bronchus-associated lymphoid tissue at bronchioles-alveolar duct junctions back onto the bronchiolar epithelium.

The literature review on effects of extra-pulmonary NPs-mediated has shown that they can mainly accumulate and retain in the liver, spleen, and lymph nodes [225-227], which may have long-term toxicity implications, and also in the heart, brain, and even in the soft tissue and bone [228]. Other studies have proved that NPs exposure caused pro-inflammatory responses in the aorta, heart, and liver [229] indirectly due to mediators “spill-over” rather than direct NP’s translocation, and uterine microvascular sensitivity, systemic microvascular dysfunction [220, 223, 230].

Biomarkers of potential NPs-induced systemic effects includes inflammatory markers measured in blood and serum, while their distribution can be evaluated using various imaging techniques.
Complete blood cell count parameters such as neutrophil, monocytes, and platelet indices have been shown to be highly sensitive markers of systemic inflammation [231-234]. These markers have been widely used to determine the severity of inflammation and as predictors in tissue injury [235], cardiovascular disease [236], hypertension [237], autoinflammatory diseases, and cancer [238].

Neutrophils are polymorphonuclear phagocytic leukocytes that migrate to inflamed/damaged tissues, where they complete their functions and ultimately are eliminated by macrophages [239]. These cells respond quickly to trap and destroy invading pathogens and foreign agents. Their migration process starts with adhesion to the endothelial surface, followed by intravascular migration, extravasation, and migration in the interstitium [240]. GM-CSF or TNF-α, two neutrophil priming agents present in the peripheral blood, may enhance neutrophil chemotaxis, extravasation, and oxidative burst production [241]. Therefore, neutrophils can play a dual role. Besides their antimicrobial function, deregulation of neutrophils and their hyperactivity can lead to tissue damage due to the accumulation of neutrophils in the vascular bed, increasing vascular permeability and promoting organ failure [242].

Monocytes are a subgroup of circulating white blood cells that originate from progenitor cells in the bone marrow and further differentiate into a range of tissue macrophages and dendritic cells, which remove pathogen-associated molecular patterns, cell debris, and foreign agents [234]. It has been reported that exposure to particles causes a systemic inflammatory response that includes stimulation of the bone marrow with subsequent increases in the release of monocytes [243]. At the site of injury/inflammation, monocytes produce inflammatory cytokines and contribute to local and systemic inflammation. In a steady-state, the patrolling anti-inflammatory monocytes patrol the vasculature to monitor pathogen-associated molecular patterns and become tissue-resident macrophages. During inflammation, they differentiate into anti-inflammatory macrophages, which repair damaged tissues [244]. It has been shown that MCP-1 and MCP-3 chemokines mediate monocyte recruitment [245]. However, the mechanism of action remains unclear.
Platelets play a critical role in tissue wounding, influencing inflammation and immune regulation [246]. They usually initiate rapid clotting, vasoconstriction, inflammation, and wound biology, leading to sterilization, tissue repair, and resolution [247]. Platelets release transmigration stimulating chemokines and cytokines that can recruit additional platelets, drive monocyte activation and differentiation, as well as neutrophil adhesion and monocyte recruitment to the endothelium [248]. Human clinical studies of concentrated ambient ultrafine and fine particles and in vitro and in vivo exposure studies to diesel exhaust particles have shown that exposure to ambient UFP activates circulating platelets [249, 250]. Platelets were linked to asthma through increased P-selectin and eosinophil β1-integrin-mediated leukocyte recruitment [251, 252].

As already mentioned above, the cytokines and chemokines secreted in the lung in response to NPs exposure and injury can cross into the systemic circulation [221]. Hence, measuring these markers in the serum could be used as an indicator of pulmonary inflammation. Indeed, changes in serum levels of pro- and anti-inflammatory cytokines have been observed in human subjects exposed to nanoparticles from photocopiers [253], diesel emissions [254], among others. These markers will not be further discussed here as they were already discussed in detail previously.

C-reactive protein (CRP) is the most sensitive acute-phase protein with potent pro-inflammatory properties, and is used extensively in clinical settings to detect incidence and the degree of inflammation [255]. It was reported that IL-6 is the main inducer of CRP gene expression, with IL-1 enhancing the effect [256]. In vitro and in vivo animal studies suggest that CRP levels increase in response to particle exposure [257].

In a clinical setting, traditionally, serum biomarkers are measured to evaluate organ damage or disease state, which could also be applied to evaluate systemic toxicity from exposure to various environmental toxicants, including NPs. Examples of serum biochemistry biomarkers and the most common abnormality indicated by the test are:

- albumin (ALB): Impaired renal and hepatic function
- alkaline phosphatase (ALKP): hepatic disease involving the biliary system
• alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH): hepatic parenchymal lesions
• creatine kinase (CK): skeletal muscle lesions attributable to trauma or vigorous exercise
• creatinine (CREA), urea (BUN), and uric acid (URIC): renal disease
• gamma-glutamyltransferase (GGT): hepatic neoplasia
• ammonia (NH3): portosystemic/liver shunt (a bypass of the liver by the body’s circulatory system)
• inorganic phosphate (PHOS): renal failure and gastroenteritis
• total bilirubin (TBIL): intrahepatic obstruction
• total protein (TP): Impaired renal and hepatic function, dehydration, gastrointestinal lesions.

However, because some markers lack the specificity and sensitivity needed to integrate these into the risk assessment process, novel improved biomarkers are needed [258].

Field emission scanning electron microscope (FE-SEM) with energy dispersive spectroscopy (EDS) is the simplest and most efficient method that allows analysis of inhaled NPs deposited in the lung or translocated to secondary organs, such as the liver and kidney [259, 260]. This method enables direct tissue examination, allowing identification and characterization of NPs, as well as alteration of the particles by processes active in the lung, thanks to improved image resolution, element detection, and analytical sensitivity.

1.3.2. Volatile organic compounds

Identifying the species present in the gaseous phase released during 3-D printing is essential in exposure assessment. It was shown that the VOCs emitted from 3-D printer operation can pose serious risk concerns on human health and the environment as some are known irritants, carcinogens, odorants, reprotoxins, etc. [110]. Even though the concentrations reported in the literature were found to be low [90, 93, 98, 108, 119, 122, 126], long-term exposure to these chemicals at low concentrations may cause cancer [261]. These are listed as hazardous air pollutants (HAPs) in the US environmental
protection agency (EPA) database [262]. The International Agency for Research on Cancer (IARC) classifies substances from the HAPs list in terms of groups of suspected carcinogens [263].

Acetaldehyde, ethylbenzene, formaldehyde, methylene chloride, styrene, and toluene were VOCs commonly detected in ABS, PLA, and nylon filaments [90, 93, 95, 107, 108, 110, 112, 119, 122, 126]. Formaldehyde is listed as a human carcinogen, whereas methylene chloride as probable human carcinogens, and lastly, acetaldehyde, styrene, ethylbenzene as possibly human carcinogens under the IARC [263] and California Proposition 65 (Prop 65) [264]. Toluene is listed under Prop 65 for its reproductive and developmental toxicity. These six VOCs are also listed under the ACGIH® TLV® [117], Committee for the Health Assessment of Building Products’s Lowest Concentration of Interest (AgBB LCI) [265], and California Department of Public Health Standard Method (CDPH SM) [266]. Other common thermal degradation byproducts of filaments listed in these health risk tables are benzenes (known human carcinogen) and aldehydes (probable human carcinogen). Davis et al. [122] determined that 51 VOCs (29%) for ABS, 36 VOCs (63%) for PLA, and 30 VOCs (64%) for nylon out of the total number of individual VOCs identified for each filament material were found at least once in the five risk tables mentioned previously.

Other hazardous chemicals released that are designated as carcinogenic to humans include trichlorethylene, acrylonitrile, methylene chloride, chloroform, tetrachloroethylene, which are defined as probably and possibly carcinogenic to humans [122, 262]. Hexane and 1,2,4-trichlorobenzene were also detected and not listed as carcinogens but are known to have effects on the central nervous system after inhalation. Chlorobenzene, 1,4-dichlorobenzene, lactide, methyl methacrylate, and hexachloro-1,3-butadiene were also detected and not classified carcinogens or toxic to the central nervous system but are hazardous pollutants that can cause narcosis, irritation, and cardiac disease [262].

Davis et al. [122] compared predicted indoor air concentrations in personal space, residential room, and classroom to relevant national and international IAQ criteria (Figure 1.7) based on the CDPH SM, 1/10 ACGIH TLV®, and AgBB ICL risk tables. For this study,
they used ABS, PLA, nylon, PVA, and HIPS filaments. For ABS, PLA, and nylon, respectively, 30 VOCs (59%), 19 VOCs (53%), 14 VOCs (47%) out of all the individual VOCs associated with known health effects were introduced from 3-D printing at much higher concentrations than background IAQ levels. Predicted concentrations of caprolactam, an irritant associated with ocular and respiratory toxicity, exceeded some personal exposure criteria and could exceed AgBB, CREL (chronic reference exposure level), and ACGIH (1/10 TLV® TWA) criteria. The predicted concentrations of formaldehyde, a known human carcinogen, exceeded some personal exposure and maximum residential exposure criteria and could exceed limits set by CDPH SM. Benzene, also a known human carcinogen, exceeded some criteria for personal exposure and could be twice the concentration of the CDPH SM's criteria for personal exposure. The maximum predicted residential concentration and the average personal exposure concentration for 2-butenal, a highly reactive aldehyde that may cause inflammation and respiratory and neurotoxic symptoms, exceeded the allowable concentration in AgBB. The maximum concentration for personal exposure for styrene, an irritant, narcotic, and neuropathic agent, was above the allowable concentration by AgBB. The maximum predicted personal exposure concentrations of benzaldehyde and acetaldehyde, both irritants and/or carcinogens, exceeded recommended AgBB and criteria in one of the three risk tables. Other VOCs that might potentially exceed the allowable indoor concentrations in the personal breathing zone were 1-butanol, xylenes, toluene, methyl styrene, and ethylbenzene. While none of the chemicals of concern showed predictions in a classroom with three operating 3-D printers that exceeded the IAQ criteria, concentrations of both caprolactam and 2-butenal criteria could be reached if 18 3-D printers with nylon filaments were to operate simultaneously in a classroom. The use of this many printers in a room is common in school/university settings.
Figure 1.7. Predicted indoor air concentrations in A: personal space, B: residential room, C: classroom compared to relevant national and international IAQ criteria. Error bar extends to the predicted concentration value using the maximum ER measured. Residential background (BG) concentrations were obtained from Logue et al. [38]and Offermann et al. [39], and classroom background concentrations from Godwin et al. [40] and Jenkins et al. [41]. Reproduced from Davis et al. [122], Characterization of volatile organic compound emissions from consumer level material extrusion 3D printers. Building and Environment, 2019. 160: p. 106209. https://doi.org/10.1016/j.buildenv.2019.106209 with permission from the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND) https://creativecommons.org/licenses/by-nc-nd/4.0/.
Stefaniak et al. [119] raised concerns about potential adverse health effects caused by VOCs’ exposure during pre-printing and post-printing operations. The researchers estimated that 50 spools of ABS filament could release about 110 and 640 μg/m³ acetaldehyde and styrene, respectively, after 4 hours of storage in a 3 m³ closet without ventilation. After the object was printed, it emitted 4.4–7.4 ng acetaldehyde g⁻¹ h⁻¹ and 5.1–5.9 ng styrene g⁻¹ h⁻¹. If hypothetically, 1000 combs are stored in a 12 m³ storage room, after 4 hr, acetaldehyde and styrene concentrations would be about 5–9 and 6–7 μg/m³. It was noted that VOC’s exposure levels were low during all steps of FDM processes, as per TLV®s and Recommended Exposure Limits (RELs). However, since these values are not intended for home environments, the user should consider using precautions, especially around children, the elderly, and sensitive individuals.

1.3.3. FFF 3-D printer emissions and printed parts toxicity studies: a literature review

Concerns were expressed regarding FFF 3-D printing, as the printer-emitted emissions/nanoparticles generated during operation have been linked to adverse health effects [101, 223, 267, 268]. A PubMed and Google scholar literature search was performed to include relevant publications from 2000 up to December 2020 that evaluated the toxicity of FFF 3-D printer emissions and of FFF 3-D printed parts/raw materials.

1.3.3.1. In vitro studies

Zhang et al. [269] collected ABS and PLA emitted-particles from a consumer-level 3-D printer on membrane filters, resuspended them in deionized water, and assessed their in vitro toxicity using rat alveolar macrophages and human tumorigenic lung epithelial cells. It was found that both ABS and PLA were cytotoxic, decreased cell viability, and increased oxidative stress and inflammatory responses. Furthermore, the oxidative potential of ABS, PLA, and nylon was assess using cell-free DTT assay. This study found that PLA had the lowest OPᵥDTT (particle oxidative potential normalized per particle mass and the volume of air sampled), followed by Nylon (OPᵥDTT was 25 times higher than of PLA), and ABS (OPᵥDTT was 60–100 higher than that of PLA).
1.3.3.2. In vivo studies

Exposure of zebrafish embryos to 3-D printer raw materials has been found to affect their survival and hatching rates, resulting in the inhibition of embryo development, estrogen receptor transactivation, and oocyte maturation [270-272]. Additionally, it was found that the stereolithography-printed parts were significantly more toxic to zebrafish embryos than FFF-printed parts and that exposure to ultraviolet light diminished their toxicity [271].

Stefaniak et al. [223] investigated the acute toxic effects of ABS 3-D printer emissions on cardiovascular function after nose-only inhalation exposure in rats. It was found that the exposure to these emissions for 3 h induced significantly higher mean arterial pressure concomitant with elevated resting arteriolar tone and impaired endothelium-dependent arteriolar dilation.

Zhang et al. [269] exposed mice by intra-tracheal administration of ABS or PLA particle suspensions extracted from filters and 24 h after exposure, analysis of inflammatory response in BALF was carried out using flow-cytometry. It was found that all particles produced a strong inflammatory response as indicated by the increase in neutrophils number. Furthermore, the PLA emitted particles produced stronger inflammatory response than ABS. However, it is important to note that in this study the filament concentrations tested were not similar.

1.3.3.3. Human studies

A case report [268] showed that a businessman with a history of childhood asthma who operated ten 3-D printers using ABS feedstock in a small work area (approximately 85 m³) developed work-related asthma within ten days of initial exposure. He experienced chest tightness, shortness of breath, and coughing. His symptoms slowly improved and eventually resolved after his workplace was modified to reduce 3-D printer emissions exposure. He replaced ABS (a high emitter filament) with PLA (a low emitter filament), reduced the number of printers utilized, and started using an air purifier with a high-efficiency particulate air filter and organic cartridge.
In a randomized, cross-over design study [273], healthy volunteers were exposed to ABS and PLA emissions from a desktop 3-D printer for 1 h. Before and after exposure, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, INF-γ) and eosinophil cationic protein in nasal secretions, exhaled nitric oxide, urinary 8-isoprostaglandin F2α (oxidative stress biomarker), and self-reported symptoms were assessed. Although no clinically significant acute changes in the biochemical responses of nasal secretions and urine were found, the volunteers’ exhaled air showed slightly increased levels of nitric oxide after ABS exposure compared to PLA, which might be due to inhaled ultrafine particle-induced eosinophilic inflammation.

In a health survey [267], about 60% of participants using 3-D printing in commercial prototyping businesses, educational institutions, and public libraries reported weekly respiratory symptoms. The same study also determined that working more than 40 h per week was significantly associated with asthma or allergic rhinitis diagnosis.

1.4. RESEARCH GAPS AND PURPOSE OF THE STUDY

The development of low-cost FFF 3-D printers has made this technology widely accessible for consumers and industry, and its use is likely to continue to increase.

Because of its enhanced product efficiency and reduced waste, FFF 3-D printing is a valuable tool for more sustainable manufacturing and, possibly, the next generation of the manufacturing process. The U.S. 3-D printing market is anticipated to grow at a compound annual growth rate of 27% by 2024. It is expected to exceed more than a $7 billion industry in the U.S. over this time [274].

As with any emerging technology, the knowledge and research about health and safety risks from exposure to FFF 3-D printer emissions is unclear and understudied. Furthermore, there are no standard analytical methods to ensure all emissions are collected uniformly for easy comparison between studies. Standardized test and analysis methods to reduce and avoid large discrepancies are needed. The exposure variability could lead to poor predictions of exposure concentrations and could, inevitably, mislead the relationship between exposure levels and adverse health outcomes.
Currently, no occupational or consumer exposure limits for 3-D printer emissions have been established. This cannot be accomplished until the potential toxicological effects of exposure to emissions are fully understood. It is already established and described in detail in the previous section that FFF 3-D printing involves heating the plastic filament to its melting point, which results in release of a mixture of volatile organic compounds (including carcinogens and chemicals that cause nervous system disorders) and ultrafine particles that can cause respiratory and cardiovascular diseases [90, 92, 93, 98, 101, 119, 126, 269, 275]. However, there are no sufficient published data regarding the toxicity of aerosolized emissions from 3-D printers to address the hazards and potential risks associated with exposure. As presented in the previous section, only five published studies have examined the health effects of inhaled FFF 3-D printer emissions, and three studies evaluating the potential toxicity of the printed parts or raw materials used in FFF. Therefore, further evaluations and research to assess the exposure to 3-D printer emissions are needed especially identifying the pulmonary adverse health effects as inhalation is the major route of human entry and exposure for hazardous toxicants such as particles and gases.

There is a critical need to fill knowledge gaps and assess the potential toxicological effects of exposure to the FFF 3-D emissions in order to establish effective control strategies and exposure limits for specific materials to prevent adverse health effects from 3-D printing emission exposure. As such, this study aims to fill the current research gap by studying 3-D printer emissions' toxicity in order to help develop and implement evidence-based exposure limits and control strategies. This study's objectives include 3-D printer emissions hazard characterization, investigation of the in vivo respiratory and systemic effects in rats exposed by whole-body inhalation, and in vitro evaluation of pulmonary effects using a conventional submerged model and a human air-liquid-interface (ALI) airway tissue model.

This study's central hypotheses is that emissions generated during 3-D printing are toxic and exposure to these emissions induces pulmonary and systemic adverse health effects. We will test the central hypothesis and accomplish the objective of this study by pursuing the following three specific aims:
**Aim 1:** To evaluate ABS and PC filaments 3-D printer emissions-induced *in vitro* cell toxicity.

**Aim 2:** To evaluate the inhalation respiratory and systemic toxicity of ABS emissions in rats.

**Aim 3:** To evaluate the ABS emissions pulmonary effects *in vitro* in a physiologically relevant air-liquid interface (ALI) model of human primary bronchial epithelial (NHBE) cell.

### 1.5. Conceptual Outline of the Dissertation

**Chapter 2: To assess acrylonitrile butadiene styrene (ABS) and polycarbonate (PC) filaments three-dimensional (3-D) printer emissions-induced cell toxicity.**

The main components of the 3-D printer emissions collection system used in this study consisted of a stainless-steel chamber to house a desktop 3-D printer, a laptop computer located outside of the chamber to control printer operation, and four BioSamplers®. The particles and vapors in chamber air were trapped in the collection medium as the air was drawn through biosamplers' orifices into the collection vessel. After each print job, the contents of the four BioSamplers® were combined, aliquots were taken for physicochemical characterization, and the remainder of the collection medium was stored at 4 °C in a sterile glass bottle and used for *in vitro* toxicity assessment. To evaluate the ABS emission-induced toxicity, small airways epithelial cells (SAEC) were seeded in 96-well plates and exposed for 24 h to six doses of ABS-emissions. To do that, the collection medium generated from two print runs were each delivered at three dilution levels: as undiluted (or 0% dilution), 25% dilution, and 50% dilution, resulting in six doses. For cellular uptake and cell morphology evaluation, SAEC were seeded in 6-well plates at a density of $0.5 \times 10^6$ cells per well and incubated overnight, followed by exposure to undiluted 3-D printer emissions in 2 mL cell culture medium for 24 h. The background sample was used undiluted. The control samples were treated with Small Airway Epithelial Cell Growth Medium (SAGM™). The endpoints measured included cellular uptake, cell viability, cell membrane damage, ROS production and apoptosis using high...
content screening, total antioxidant capacity, glutathione peroxidase levels in cell lysates, and cytokines and chemokines released in cell supernatants.

**Chapter 3: To assess pulmonary and systemic toxicity in rats following whole-body inhalation exposure of 3-D printer emissions from acrylonitrile butadiene styrene (ABS) filament.**

To date, the only published study evaluating the effects of inhaled ABS-emissions [223], investigated the acute toxic effects on cardiovascular function via a nose-only inhalation in rats. It was found that exposure to 1 mg/m³ ABS-emission for 3 h induced significantly higher mean arterial pressure concomitant with the elevated resting arteriolar tone and impaired endothelium-dependent arteriolar dilation. These results indicated that the 3-D printer emissions could potentially induce systemic toxic effects, similar to what was described previously concerning the inhalation of other types of nanoparticles and ultrafine particulate matter [276-278]. This study focused on ABS emissions-induced lung responses, and potential involvement in transducing systemic effects after pulmonary insult based on the Brook et al. [221] proposed “systemic spill-over” mechanism that proinflammatory mediators travel from the lung into circulation.

The current study was designed with the objective of identifying and characterizing potential hazards from FFF 3-D printing and provide data for quantitative inhalation risk assessments for the development of health-based exposure limits for the use of ABS thermoplastic filament (the most popular filament on the marker and in the industry), as currently, no exposure limits exist.

An inhalation exposure system was specifically designed and constructed to deliver FFF 3-D printer emissions (NPs + VOCs) to a whole-body rodent exposure chamber. Sprague-Dawley rat strain (Hla(SD)CVF) was chosen as animal model because is a preferred animal species to characterize and evaluate the toxicologic potential of new hazardous substances by the National Institute for Occupational Safety and Health (NIOSH) and National Toxicology Program. Also, the previous work in the biomedical literature validates the use of this rat strain as an animal model for lung toxicology studies.
The following measures of pulmonary injury and inflammation, as well as systemic effects, were conducted on ABS-exposed and filtered-air (negative control) animals at all time points. SEM images of lungs from animals exposed to ABS-emissions were taken to confirm that the ABS particle reached and deposited in the alveolar region. To determine the cellular uptake of particles and morphological changes, TEM analysis of BAL cells was carried out. For the assessment of pulmonary damage, total lung cells as well as differentiated cells in BAL fluid (BALK) were counted. To further examine the pulmonary injury, the lactate dehydrogenase (LDH) activity, total protein, surfactant protein A (SP-A) and D (SP-D) levels in BALK were measured. To evaluate inflammatory responses, a panel of 9 pro- and anti-inflammatory cytokines were quantified. Along with inflammation, oxidative stress is a key mechanism linking particle exposure to observable physiological and biochemical responses. As such, protein carbonyl and malondialdehyde (MDA) levels were measured to determine the extent of the oxidative damage of protein and lipid oxidation after exposure to ABS emissions, respectively. To evaluate whether the pulmonary inflammation triggered by exposure to ABS-emissions would cause systemic toxic effects to secondary organs, blood samples were analyzed for the clinical hematological parameters, and the biomarkers of muscles, metabolic, renal, and hepatic function were evaluated. Furthermore, the levels of pro- and anti-inflammatory cytokines (similar panel as assessed in BALK) in serum were measured. To confirm the particle-induced injury, sections of lung and nasal passages (T1, T2, T3, and T4) were reviewed for lesions by a board-certified veterinary pathologist.

Chapter 4: To evaluate 3-D printer emissions pulmonary effects in a physiologically relevant air-liquid interface (ALI) model of human primary bronchial epithelial (NHBE) cell.

Considering the dosimetry drawbacks and lack of the physiological features of airway mucosa when using submerged in vitro models as well as challenging data extrapolation from animals to humans due to respiratory tract interspecies variability, in this study normal, human-derived bronchial epithelial cells (NHBEs) cultured at the air-liquid interface (ALI), were used to further test the respiratory toxicity of ABS FFF 3-D printer emissions. Moreover, given that there are numerous types of filament on the
market, and their toxic effects may vary as their emissions’ physicochemical characterization varies with each type, and within each type varies with color [90, 93, 101, 119] it is very expensive and practically impossible to test all the filaments using an *in vivo* model.

Primary NHBEs cultured at ALI is promising in the domain of inhalation toxicology as it mimics the *in vivo* respiratory tract in its organization and stratification, and avoids limitations posed by the submerged monolayer cultures. Moreover, we will provide new tools for investigating the effects of FFF thermoplastic filaments, as alternatives to animal testing. Physiologically relevant respiratory mucosa models, such as *in vivo*-like *in vitro* models cultured at ALI, have been established and are being routinely used to study inhaled particles or gases mediated respiratory toxicity.

In order to assess the effects of ABS emissions on NHBE 3D culture, a series of examinations were conducted such as: H&E and periodic acid-Schiff (PAS) staining, immunofluorescence for α-tubulin, E-cadherin, MUC5AC, and ZO-1 staining of epithelium on fixed membranes, and trans-epithelial electrical resistance (TEER), and ciliary beat on live epithelium. Afterward, cytotoxicity, tissue injury, and inflammatory and immune system regulation markers were evaluated in the supernatant.

**Chapter 5: General discussion.**

General conclusions on the body of work will be discussed here as well as future directions to carry the project forward.
CHAPTER 2: ACRYLONITRILE BUTADIENE STYRENE (ABS) AND POLYCARBONATE (PC) FILAMENTS THREE-DIMENSIONAL (3-D) PRINTER EMISSIONS-INDUCED CELL TOXICITY


PMID: 31562913
DOI: 10.1016/j.toxlet.2019.09.013
ABSTRACT
During extrusion of some polymers, fused filament fabrication (FFF) 3-D printers emit billions of particles per minute and numerous organic compounds. The scope of this study was to evaluate FFF 3-D printer emission-induced toxicity in human small airway epithelial cells (SAEC). Emissions were generated from a commercially available 3-D printer inside a chamber, while operating for 1.5 h with acrylonitrile butadiene styrene (ABS) or polycarbonate (PC) filaments and collected in cell culture medium. Characterization of the culture medium revealed that repeat print runs with an identical filament yield various amounts of particles and organic compounds. Mean particle sizes in cell culture medium were 201 ± 18 nm and 202 ± 8 nm for PC and ABS, respectively. At 24 h post-exposure, both PC and ABS emissions induced a dose-dependent significant cytotoxicity, oxidative stress, apoptosis, necrosis, and production of pro-inflammatory cytokines and chemokines in SAEC. Though the emissions may not completely represent all possible exposure scenarios, this study indicate that the FFF could induce toxicological effects. Further studies are needed to quantify the detected chemicals in the emissions and their corresponding toxicological effects.

Keywords: emerging technologies, printer emitted nanoparticles, in vitro toxicity, and inflammatory response.
2.1. INTRODUCTION

Additive manufacturing (AM) is a family of processes used to build physical objects from a computer-aided design model. Fused filament fabrication (FFF) is a type of material extrusion technology used by some 3-D printers to melt and extrude a thermoplastic filament through a heated nozzle to deposit material on a plate, layer-by-layer, to create a physical object. 3-D printing provides a novel approach for the fabrication of intricate items that can potentially combine optical, chemical, electronic, electromagnetic, fluidic, thermal, and acoustic features [279]. Some of the uses of 3-D printing include tissue and organ fabrication; creating prosthetics, implants, and anatomical models; pharmaceutical research concerning drug discovery, delivery, and dosage forms; parts for motor vehicles and rocket engines; and devices in various industries to advance innovation, production, and efficiency. The U.S. 3-D printing market is anticipated to grow at a compound annual growth rate of 27% by 2024, and it is expected to exceed more than a $7 billion industry in the U.S. over this time [274].

The feedstock materials used in AM systems can be liquid or solid in the form of plastics, metals, or ceramics. Plastics used in FFF 3-D printer filaments may also include infill materials, such as wood fiber, carbon fiber, silica, or engineered nanomaterials (e.g., multi-walled carbon nanotubes). Several types of feedstock filaments are commercially available for use in FFF 3-D printers, including, but not limited to, polycarbonate (PC) and acrylonitrile butadiene styrene (ABS). Previous studies have shown that: 1) thermal degradation of these filaments during 3-D printing releases fine and ultrafine particles; 2) average particle emission rates range from $\sim10^8$ to $\sim10^{11}$ particles/min with some variances among different types and brands of filaments and within the same filaments, but different colors; and 3) particle emission rates fluctuate over the course of printing, with the highest emission rates occurring at the beginning of printing [86, 89-93]. In addition to particles, FFF 3-D printers also emit numerous volatile, semi-volatile, and non-volatile organic compounds during thermal processing, such as aldehydes, ketones, alcohols, aromatics (benzene, toluene, ethylbenzene, xylenes, styrene), and phthalates.

Several recent studies have shown that exposure to 3-D printer-emissions can induce potential toxic effects in humans and animals [223, 268, 270, 271]. A case report
showed that a businessman who operated 3-D printers using ABS feedstock in a small work area of approximately 85 m³ developed work-related asthma within 10 days of initial exposure [268]. In a human exposure study, volunteers were exposed to emissions from a desktop 3-D printer for 1 h, followed by assessing changes in acute inflammatory biomarkers [273]. Although no clinically significant acute changes in the biochemical responses of nasal secretions and urine were found, the volunteers’ exhaled air showed increased levels of nitric oxide, which might be due to inhaled ultrafine particle-induced eosinophilic inflammation [273]. Exposure of zebrafish embryos to 3-D printer raw materials has been found to affect their survival and hatching rates, resulting in the inhibition of embryo development, estrogen receptor transactivation, and oocyte maturation [270-272]. Stefaniak et al. [223] investigated the acute toxic effects of ABS 3-D printer emissions on cardiovascular function after nose-only inhalation exposure in rats. It was found that the exposure to these emissions for 3 h induced significantly higher mean arterial pressure concomitant with elevated resting arteriolar tone and impaired endothelium-dependent arteriolar dilation.

Considering the fast-growing application of 3-D printing technology, the reported emissions, and the potential adverse health effects from exposure to these emissions, there is a need to study the toxicity of 3-D printer emissions and the mechanisms of toxicity both in vivo and in vitro. In this study, emissions were generated in a 0.62 m³ environmental chamber using a commercially available FFF 3-D printer using PC or ABS filaments. The emissions were collected in a serum-free cell culture medium and characterized to determine the mean hydrodynamic particle size (nm), particle concentration (particles/mL), zeta potential, and small organic molecule content. Human small airway epithelial cells (SAEC) were exposed to these emissions in cell culture medium to investigate cytotoxicity, apoptosis, and oxidative stress and inflammatory markers at 24 h post-exposure.

2.2. MATERIALS AND METHODS

2.2.1. Emissions collection setup

The 3-D printer emissions were collected in a stainless-steel test chamber (TSE Systems, Chesterfield, MO). The collection system consisted of 1) a 0.62 m³ stainless-
steel chamber to house a desktop 3-D printer (LulzBot Mini, Aleph Objects, Inc., Loveland, CO); 2) a laptop computer located outside of the chamber to control printer operation; 3) four BioSamplers® (SKC, Inc., Eighty Four, PA) containing 20 mL of Small Airway Epithelial Cell Basal Medium (SABM™) (Lonza Inc., Allendale, NJ) (located inside of the chamber at a distance of 20 – 25 cm from the printer); and 4) tubing that connected the BioSamplers® to high flow pumps (located outside the chamber) to sample chamber air at 12.5 L/min (via sonic orifices in the BioSamplers®). The purpose of the chamber was to collect sufficient amounts of emissions for the planned *in vitro* studies in a reasonable time period. As such, the chamber volume is smaller than a typical room where a 3-D printer may be operated; however, the distance from the BioSampler® inlet to the printer is reasonable based on our experiences performing workplace assessments. Each BioSampler® consists of an inlet section, a nozzle section containing three nozzles, and a collection section containing SABM™. The three sampler components are connected by a fritted glass fitting. When connected, the three nozzles are positioned above SABM™ in the collection vessel and airflow through these nozzles (flow rate controlled by sonic orifices) creates a vortex. As air is drawn through the nozzles, particles and vapors in chamber air are trapped in the SABM™. To minimize evaporation of the cell culture medium, two BioSamplers® were operated for the first 45 min of the print job, and two BioSamplers® were operated during the last 45 min of the print job. Upon completion of sampling, each BioSampler® (each containing about 10 mL of unevaporated media) was rinsed with a few milliliters of fresh media, and the contents from all four BioSamplers® were combined in order to obtain an approximately 50 mL sample that was representative of emissions from the entire 1.5-h print job. In preliminary studies performed to evaluate the potential effect of temperature on emissions collection, chamber air was sampled using BioSamplers® placed in ice baths or at room temperature; no difference was observed in collected emissions characteristics between the two methods. A two-piece high-efficiency particulate air filter and activated carbon filter (Whatman, Maidstone, UK) was attached to the chamber inlet to remove particles and organic chemicals, respectively, from the ambient air before it entered the chamber. The chamber has sampling ports through which sampling tubing was inserted to connect to the high flow pumps (*Supplemental Figure 2.1* illustrates the sampling setup). The 3-D
printer sat on an approximately 100 cm x 60 cm perforated stainless-steel support plate to allow for upward airflow inside the chamber. This minimized areas of stagnant air in the chamber and resulted in 3-D printer emissions being distributed uniformly in this chamber; thereby, minimizing bias relative to sampling positions. The chamber leak rate and air exchange rate were determined by dosing the chamber with sulfur hexafluoride (SF₆) and monitoring its concentration using an infrared spectrophotometer (Miran Sapphire, Thermo Scientific, Waltham, MA). The leak rate over an eight-hour period was found to be 0.8%, and the air-change rate was 2.42 air changes per hour [280, 281].

Black PC (Gizmo Dorks LLC, Temple City, CA) and black ABS (3DXTech, Byron Center, MI) filaments were used to 3-D print an artifact designed by the National Institute of Standards and Technology (NIST) [282]. Printer settings were 240 °C/110 °C (extruder nozzle temp/printer bed temp) and 290 °C/110 °C for ABS and PC, respectively (as recommended by manufacturers). The NIST artifact was printed three times using PC filament (runs designated PC1, PC2, and PC3) and three times using ABS filament (runs designated ABS1, ABS2, and ABS3) and was scaled to print within 1.5 h. A background sample was also collected for 1.5 h by sampling chamber air, while the 3-D printer was inside the chamber but powered off and not operating. After printing, aliquots of culture media were taken from the samples for characterization (described below). The remainder of the medium was stored at 4 °C in a sterile glass bottle and used for in vitro toxicity assessment within 24 – 36 h.

2.2.2. Emissions characterization

2.2.2.1. Particle size and concentration in cell culture medium

Mean hydrodynamic particle size and particle concentration were determined using nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern Instruments, Worcestershire, UK). For analysis, a syringe pump was used to inject all samples through a Low Volume Flow Cell top plate at a constant rate at room temperature. Camera and threshold settings in the NTA instrument varied slightly with each sample to ensure accurate particle characterization. SABM™ and background samples were also ran at camera levels/threshold settings similar to the ABS and PC printing samples in an effort
to determine how much the SABM™ and ambient air contributed to the NTA measurements. Each sample was captured five times for 60 seconds, for a total capture time of 5 minutes per sample.

Zeta potential was determined by electrophoretic light scattering (ELS) using a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). The pH of each sample was measured before each run using a SevenMulti calibrated electrode (Mettler-Toledo, LLC, Columbus, OH). The parameters of all runs were as follows: refractive index of material = 0.100, absorbance of material = 0.100, refractive index of dispersant = 1.334,

2.2.2.2. Morphology and elemental composition of particles in medium

Particles collected in cell culture medium were filtered onto a 0.2-micron polycarbonate filter, mounted on a clean aluminum sampling stub, and coated with a thin layer of gold/palladium. Particles collected on the filters were imaged and analyzed using a field emission – scanning electron microscope (FE-SEM, Hitachi S-4800, Tokyo, Japan) equipped with an energy dispersive x-ray detector (EDX, Quantax, Bruker Scientific Instruments, Berlin, Germany) to determine elemental contents.

2.2.2.3. Analysis of organic compounds collected in the cell culture medium

Samples were stored at 4 °C until ready for analysis when they were brought to room temperature. An unfiltered 2-mL aliquot was sampled by submerging a polydimethylsiloxane/divinylbenzene (PDMS/DVB) (65 µm film thickness) solid-phase microextraction (SPME) fiber in the solution for one hour. After one hour, the SPME fiber was removed, and the sample was analyzed by gas chromatography-mass spectrometry (GC-MS). The inlet temperature was at 250 °C in splitless mode. The GC-MS was operated in constant flow mode at 1.2 mL/min with ultra-high purity grade helium as the carrier gas. Separation occurred using an HP-5MS column and the following oven program: 40 °C for 6 min, ramp to 80 °C at 20 °C/min, hold 4 min, ramp to 240 °C at 20 °C/ min, hold 2 min. Compounds were tentatively identified by comparing mass spectra to the 2014 NIST/EPA/NIH Mass Spectral Library (NIST 14); only compounds that matched ≥70% with the Library are reported herein.
2.2.3. In vitro toxicity evaluation of collected 3-D printer emissions

2.2.3.1. Quantitation of endotoxin

Potential endotoxin contamination was examined using Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Waltham, MA). A standard curve representing endotoxin unit/ mL (EU/ mL) was created using E. coli endotoxin as a standard, according to the manufacturer’s instructions. The level of endotoxin for all samples was similar to the endotoxin-free water sample, which was less than 0.05 EU/mL (approx. 0.005 ng/mL).

2.2.3.2. Cell culture

Human SAEC were obtained from Dr. Tom K. Hei’s laboratory [283] at Columbia University (New York, NY, USA) and maintained in SABM™ supplemented with the Small Airway Epithelial Growth Medium (SAGM™) BulletKit, at 37 °C with 5% CO₂, according to the manufacturer’s instructions (Lonza Inc., Allendale, NJ).

To assess cytotoxicity, oxidative stress response, apoptotic effects, and cytokine production, SAEC were seeded in 96-well plates at a density of 1.5 x 10⁴ cells per well overnight, followed by serum-starvation for 12 h. This was done to model an epithelial monolayer response. Next, SAEC were exposed for 24 h to 100 μL/ well of medium containing PC or ABS emissions. Each PC (prints 2 and 3) and ABS (prints 2 and 3) emissions collected in the medium were mixed by vigorous vortexing for 5 min, and exposed as undiluted (0%), 25% dilution, and 50% dilution in serum-free SABM™, resulting in six doses for each filament type (Supplemental Table 2.1). For cellular uptake and cell morphology evaluation, SAEC were seeded in 6-well plates at a density of 0.5 x 10⁶ cells per well overnight, followed by exposure to undiluted 3-D printer emissions in 2 mL cell culture medium for 24 h. The background sample was used undiluted. The control samples were treated with SABM™.

2.2.3.3. Cell viability assay

Cellular viability was determined using the AlamarBlue bioassay according to the manufacturer’s instructions (ThermoFischer Scientific, Waltham, MA). Briefly, at 24 h
post-exposure, the supernatants were collected for the measurements of lactate dehydrogenase (LDH), along with cytokine and chemokines release. The cells were rinsed with 100 μL SABM™/ well once, followed by the addition of 200 μL of 10% AlamarBlue solution to each well. After 4 h incubation at 37 °C, the viability was measured by quantifying fluorescence levels (ex/em 560/590 nm) using the Synergy H1 hybrid multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT).

2.2.3.4. Cell membrane damage

The release of LDH into the cell-free supernatants was assayed spectrophotometrically by monitoring the reduction of nicotinamide adenine dinucleotide at 340 nm in the presence of lactate using a Lactate Dehydrogenase Reagent Set (Pointe Scientific, Inc., Lincoln Park, MI).

2.2.3.5. Transmission electron microscopy

To evaluate the cellular uptake of particles and morphological changes, exposed SAEC were washed with HEPES Buffered Saline solution and detached using Trypsin/EDTA Solution (Reagent Pack™ Subculture Reagents, Lonza). After cells were released, the trypsin was neutralized with Trypsin Neutralizing Solution (Lonza) and harvested by centrifugation at 800 × rpm for 5 min at room temperature, followed by fixation in Karnovsky’s fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1M Sodium Cacodylic buffer). Then, the cells were post-fixed in osmium tetroxide, mordanted in 1% tannic acid, and stained en bloc in 0.5% uranyl acetate. The samples were embedded in epon™ (epoxy resin in xylene), sectioned, and stained with 4% uranyl acetate and Reynold’s lead citrate. The sections were imaged on a JEOL 1400 transmission electron microscope (JEOL, Tokyo, Japan).

2.2.3.6. Biomarkers of oxidative stress in cell lysates

Oxidative damage was evaluated using Total Antioxidant Capacity (TAC) and Glutathione Peroxidase (GPx) assay kits (Abcam, Cambridge, CA) with minor modifications. After the conditioned media were collected, the cells were re-suspended in 50 µL MilliQ water and 100 µL assay buffer, respectively, and stored at -80 ºC for 24 h to
induce lysis. Upon thawing, the plates were vigorously shaken at 900 rpm for 5 min, followed by pipetting up and down three times, and then transferred into the test tubes. Both assays were performed according to the manufacturer’s instructions. The results were normalized to total protein concentrations determined using Pierce™ BCA Protein Assay (ThermoFisher Scientific, Waltham, MA).

2.2.3.7. High content screening

2.2.3.7.1. Reactive oxygen species production

For high content screening (HCS), live cells were stained with a cocktail consisting of 5 µM CellROX™ Deep Red reagent, 5 µg/mL of CellMask™ Green plasma membrane stain, and 1 µM Hoechst 33342 fluorescent nuclear stain (Thermo Fisher Scientific, Waltham, MA) in culture medium, and incubated for 30 min at 37 °C. Next, the plates were washed two times with warm HEPES Buffered Saline Solution (Lonza) and imaged immediately on an ImageXpress Micro XLS (IMX) system (Molecular Devices, Sunnyvale, CA). Nine sites per well were imaged at 20X magnification with a CMOS 16-bit digital camera using Cy5 (ex/em 628/692 nm), TRITC (ex/em 543/593 nm), and DAPI (ex/em 377/447 nm) filter cubes. Images from HCS assays were analyzed with the MultiWavelength Application Module in MetaXpress v5.3 software to determine mean ROS intensity [284]. Briefly, masks were applied to cell nuclei, plasma membrane staining, and ROS signal by user-defined parameters including minimum width, maximum width, minimum intensity above background, and minimum area stained. Only those positively stained masks that contained a nucleus were scored as a cell. Mean integrated intensity of cellular ROS stain was calculated for each site by determining stain intensity under the area of each mask divided by the number of cells in each image. Mean intensities were then averaged within and across replicates.

2.2.3.7.2. Apoptosis and necrosis

The apoptotic and necrotic cells were determined using Cell Meter™ Apoptotic and Necrotic Multiplexing Detection kit (AAT Bioquest, Inc., Sunnyvale, CA) according to the manufacturer’s instructions. Briefly, at 24 h post-exposure, a cocktail consisting of 2 µL of Apopxin™ Red apoptosis stain, 1 µL of 200X Nuclear Green™ DCS1 necrotic stain,
and 1 µL of CytoCalcein™ Violet 450 cytoplasmic stain was added to each well in culture medium and incubated for 30 min at 37 °C. Next, the plates were washed twice with warm HEPES Buffered Saline Solution and imaged immediately on the IMX system. Apopxin™ Deep Red staining on the plasma membrane indicated dye binding to phosphatidylserine, an indicator of the apoptosis. Nine sites per well were imaged at 10X magnification using Cy5, FITC (ex/em 470/525 nm), and DAPI filter cubes. To analyze the images, masks were applied using minimum/maximum diameters and minimum intensities for each channel as described above. Apoptotic cells appeared red and green + red, the necrotic cells were green, and viable cells appeared blue only. Cell counts and percentages for each phenotype at each site were calculated and averaged within and across replicates.

2.2.3.8. Cytokines, chemokines, and growth factors

The levels of IL-1β, IL-6, IL-8, TNF-α, IL-12/IL-23p40, IL-16, IL-1α, and IL-13 in the cell-free supernatants were determined using V-PLEX Cytokine and Chemokines Human Kits from Meso Scale Discovery (MSD, Meso Scale Discovery). Plates were read using MSD QuickPlex SQ 120 (Meso Scale Discovery) for electrochemiluminescence. Sample concentrations were derived from a standard curve plotted using a four-parameter logistic fit using MSD Workbench software. Data were normalized to total cell number per well determined using HCS.

2.2.4. Statistics

Studies were performed in three independent experiments with 3 - 6 replicates each. The data are represented as percent of control. All statistical analyses were performed in either SAS v9.4, or JMP v13. Mixed model regression analyses were performed on the measured variables using particle number*10^-4 as the independent variable, and printer batch as a random factor. These analyses included background levels of particles as data points, as well as zero particle controls. Regression coefficients which are significantly different from 0, indicate dose-responsiveness of the dependent variables. Analyses were performed for the two filaments separately. All analyses are considered significant at p < 0.05.
2.3. RESULTS

2.3.1. Physicochemical characterization of FFF 3-D printer emissions in cell culture medium

3-D printer emissions were collected in SABM™ and characterized to determine particle concentration, mean hydrodynamic diameter size, zeta potential, particle morphology, and organic compounds content. **Supplemental Table 2.2** presents characteristics of the SABM™ medium for the PC and ABS print runs. The printing of both filaments resulted in higher numbers of particles in cell culture medium compared to the background air control. Note that the “background” sample was collected by pulling chamber air through the BioSamplers® with SABM media while the 3-D printer was off. The background particle counts in media may be from 1) particles in the chamber or in chamber air that passed through the HEPA-filtered make-up air (usually <500 particles/cm³); 2) solids in the culture media; and/or, 3) air bubbles formed during sampling from agitation and swirling of the media in the BioSampler®. During extrusion of ABS and PC, peak particle number concentrations are $10^4$ to $10^5$ particles/cm³ [92, 285], meaning that the contribution of ambient particles to printer emitted particles is at most 5%. NTA of media (no air pulled through it) confirmed that the media contained virtually no particles indicating that there were no solids in the media that were incorrectly counted as particles. Hence, the background “particle” concentration in the SABM media presented in **Supplemental Table 2.2** consists of a small fraction of ambient dust particles, but the majority of “particle” counts were likely an artifact from the instrument sensing air bubbles formed by agitation and swirling of media while drawing air through the BioSamplers®. PC filament led to a higher number of particles compared with ABS. The mean diameters of particles of PC and ABS in the medium were 201±18 nm and 202±8 nm, respectively. The zeta potentials of particles in both suspensions were negative, about −18 mV. The number-based size distribution of the particles showed that the PC run had mainly one large distinct mode between 140-170 nm, while ABS run had two modes, the first at about 140-150 nm and the second at around 250-300 nm, suggesting that particles from ABS had a broader distribution than those from PC (**Figure 2.1**).
Representative images of 3-D printer emissions particles collected in cell culture medium indicating surface morphology (FE-SEM) and elemental composition (EDX) are shown in Figure 2.2. Occasionally particles composed of silicon were observed on the background filter that may be from the low level of particles in the filtered make-up air or a contaminant on the filter. The morphology of the background particle was more angular than the printer emitted particles, and its elemental composition was consistent with ambient dust and dissimilar from the printer emitted particles. EDX analysis showed that the main elements of PC and ABS particles were C, O, Ca, Na, Si, Ni, Cr, Fe, S, Al, and Cl (Figure 2.2).

SPME/GC-MS was used to identify the organic compounds in the collected emission medium. The results were compared with the NIST/EPA/NIH Mass Spectral Library, and the compounds that matched ≥ 70% with the library are shown in Supplemental Figure 2.2. Supplemental Table 2.3 shows for information only the qualitative results of the detected individual compounds from all print runs. Representative chromatograms in Supplemental Figure 2.2 show the retention times for each compound identified during the PC and ABS print runs (no organic compounds were detected in the background sample). During the PC print run, the compounds observed in the sample collection medium were tentatively identified as bisphenol A (BPA), p-isopropenylphenol, and phenol; whereas, styrene, 3-cyclohexen-1-ylbenzene, α,α-dimethylbenzenemethanol, and acetophenone were detected when the ABS filament was used.
Figure 2.1. Number-based size distribution of 3-D printer-emitted particles collected in the cell culture medium. 3-D printer emissions using PC (A) and ABS (B) were collected in cell culture medium, followed by analysis using NTA.
Figure 2.2. Representative images of 3-D printer-emitted particles collected in the cell culture medium indicating surface morphology and elemental composition. 3-D printer emissions were collected in cell culture medium, passed through a track-etched polycarbonate filter, and analyzed using FE-SEM (A-C) and EDX (D-F). (A) Background, (B) PC, (C) ABS. D-F are representative elemental analysis spectra for A-C. Magnification at 70k× for all images, scale bar at 400 nm.
2.3.2. Toxicological evaluation of 3-D printer emissions collected in media

2.3.2.1. Uptake of 3-D printer-emitted particles by SAEC

The uptake of particles in the medium by SAEC was investigated. The cells were cultured to confluence in 6-well plates and then were exposed to PC or ABS emissions in suspension, followed by TEM analysis. **Figures 2.3** presents TEM images indicating the internalization of PC and ABS 3-D printer-emitted particles that were enclosed in a lipid membrane, similar to an endosome.

2.3.2.2. Cytotoxicity

Both cell viability assays and LDH measurements were performed to determine cellular toxicity from exposure to emissions collected in media. The AlamarBlue-based cell viability assay revealed changes in cell proliferation, while the LDH measurements in the supernatants showed the release of a soluble cytosolic enzyme upon cell death due to cell membrane damage. 3-D printer emitted particles collected in media induced a significant ($p < 0.0001$) dose-dependent decrease in cell proliferation (**Figure 2.4, Table 2.1**). Results revealed that for each $10^4$ PC and ABS particle exposure, the cell viability decreased by 0.048% (**Table 2.1**). The decrease of cell viability correlated with the dose-dependent increase in LDH activity ($p < 0.0001$) in the supernatants. Specifically, each $10^4$ PC and ABS particle exposure resulted in a significant dose-dependent increase by 0.051% and 0.110% LDH levels in SAEC, respectively (**Figure 2.4, Table 2.1**). Taken together, these results demonstrate that exposure to both PC and ABS emissions collected in cell culture medium showed dose-dependent toxicity in SAEC.
Figure 2.3. Uptake by SAEC of 3-D printer-emitted particles collected in the cell culture medium. SAEC were treated with medium containing 3-D printer emissions for 24 h, followed by TEM image analysis. (A) Control, (B) Background, (C) 100% PC, (D) C inset, (E) 100% ABS, and (F) E inset. A, B, and C images are at 2,000x magnification, and image E at 2,500x magnification. D and F images are at 15,000x magnification. A, B, C, and E scale bar at 2 μm; D, F scale bar at 400 nm.
Figure 2.4. Cytotoxicity of the 3-D printer-emitted particles collected in the cell culture medium: cell viability and LDH activity following exposure to PC (A) and ABS (B) emissions. Experiments were performed in three independent experiments with n = 6 replicates each. The regressions lines illustrate a significantly (p < 0.0001) decreased dose-response relationship between cell proliferation and the numbers of the PC and ABS 3-D printer-emitted particles, which correlated with a significant (p < 0.0001) dose-dependent increase in the LDH activity in the supernatants. The shaded area represents the 95% confidence interval around the regression line.
Table 2.1. Linear regression slopes (with standard errors) of the all variable investigated following exposure to PC and ABS 3-D printer-emitted particles collected in the cell culture medium. All statistical analyses were performed in either SAS v9.4, or JMP v13. Mixed model regression analyses were performed on the measured variables using particle number\(^{*}10^{-4}\) as the independent variable, and printer batch as a random factor. Regressions coefficients which are significantly different from 0, indicate dose-responsiveness of the dependent variables. Analyses were performed for the two filaments separately. All analyses are considered significant at \(p < 0.05\) (*).
2.3.2.3. ROS production and oxidative stress

It has been well-established that exposure to particulate matter induces the production of ROS, which causes cell injury or oxidative stress, leading to the development of various diseases [142, 286]. SAEC were exposed to PC or ABS 3-D printer emissions collected in cell culture medium for 24 h, followed by high content imaging to determine the production of ROS. Both PC and ABS particles induced a significant (p < 0.0001) dose-dependent increase in the production of ROS (Figure 2.5, Table 2.1). The mixed model regression analyses revealed that for each 10⁴ particle exposure, ROS production increased by 0.075% and 0.148% for PC and ABS in SAEC, respectively (Table 2.1).

One of the main properties of ROS is to cause cellular injury, and cellular systems are protected from ROS-induced injury by an array of anti-oxidant defenses. Measurements of TAC and GPx activity can indirectly reflect ROS-induced oxidative stress in cells. As shown in Figure 2.6, PC and ABS emitted-particles collected in cell culture medium triggered a significant dose-dependent decrease (p < 0.0001) in TAC levels, with concomitant decline in GPx activity (Table 2.1). Specifically, for each 10⁴ particle exposure, the TAC levels decreased by 0.007% and 0.039% for PC and ABS in SAEC, respectively, whereas the GPx activities decreased by 0.016% and 0.035% for PC and ABS in SAEC, respectively (Table 2.1).

2.3.2.4. Apoptosis and necrosis

To confirm apoptosis from exposure to PC and ABS emissions collected in media, the measurement of externalization of phosphatidylserine, a hallmark of apoptosis, was applied. Results illustrated that for each 10⁴ PC and ABS exposure, the number of apoptotic cells increases by 0.235% and 0.144% in SAEC, respectively (Table 2.1). To further identify other mechanism-involved cell death, 7-amino actinomycin D (7-AAD), a membrane impermeant dye which is bound to double-stranded DNA of non-viable cells and excluded from viable cells, was used to determine necrosis upon exposure to emissions collected in media. Results reveal a significantly increased dose-response relationship (p < 0.0001) between apoptotic or necrotic events and the numbers of the
PC and ABS 3-D printer-emitted particles collected in the cell culture medium (Figure 2.7, Table 2.1). Results showed that for each $10^4$ PC and ABS particles, the number of necrotic cells increases by 0.285% and 0.375% in SAEC, respectively (Table 2.1).

2.3.2.5. Cytokine and chemokine responses

A panel of 8 pro-inflammatory cytokines and chemokines in the supernatants was evaluated to determine the inflammatory responses upon exposure to 3-D printer-emitted particles collected in medium and exposed to SAEC. With the exception of IL-8 and TNF-α in the collected ABS emissions-exposed cells, model regressions analysis (Table 2.1) reveals a significant (p<0.0001) positive correlation between the levels of IL-12p70, IL-13, IL-16, IL-1β, IL-1α, IL-6, IL-8, and TNF-α levels and the quantity of particles in the ABS and PC emissions sample medium. Details of cytokines/chemokines changes are given in Supplemental Table 2.4.
**Figure 2.5.** ROS production following exposure to 3-D printer-emitted particles collected in the cell culture medium. SAEC were stained with a cocktail consisting of 5 µM CellROX™ Deep Red reagent, 5 µg/mL of CellMask™ Orange plasma membrane stain, and 1 µM Hoechst 33342 fluorescent nuclear stain. (A) Representative confocal images of untreated cells, and cells exposed to background, PC, and ABS collected emissions for 24 h. Quantitative ROS levels of PC (B) and ABS (C) using high content screening. Magnification at 20× and scale bars are at 50 µm for all images. Experiments were performed in three independent experiments with n = 6 replicates each. The regressions lines illustrate a significantly (p < 0.0001) increased dose-response relationship between ROS production and the numbers of the PC and ABS 3-D printer-emitted particles. The shaded area represents the 95% confidence interval around the regression line.
Figure 2.6. Oxidative stress response following 3-D printer-emitted particles collected in the cell culture medium: total antioxidant capacity and glutathione peroxidase activity following exposure to PC (A) and ABS (B) emissions. Experiments were performed in three independent experiments with n = 3 replicates each. The regressions lines illustrate a significantly (p < 0.0001) decreased dose-response relationship between total antioxidant capacity and glutathione peroxidase activity and the numbers of the PC and ABS 3-D printer-emitted particles. The shaded area represents the 95% confidence interval around the regression line.
Figure 2.7. 3-D printer-emitted particles collected in the cell culture medium increase apoptosis and necrosis. SAEC were stained with a cocktail consisting of 2 μL of Apopxin™ Red apoptosis stain, 1 μL of 200× Nuclear Green™ DCS1 necrotic stain, and 1 μL of CytoCalcein™ Violet 450 cytoplasmic stain. (A) Representative confocal images of untreated cells, and cells exposed to background, PC, and ABS collected emissions for 24 h. Quantitative apoptosis and necrosis analysis of PC (B) and ABS (C) using high content screening image analysis. Magnification at 10× and scale bars are at 50 μm for all images. Experiments were performed in three independent experiments with n = 6 replicates each. The regressions lines illustrate a significantly (p < 0.0001) increased dose-response relationship between apoptotic or necrotic events and the numbers of the PC and ABS 3-D printer-emitted particles. The shaded area represents the 95% confidence interval around the regression line.
2.4. DISCUSSION

The toxicity evaluation of emissions generated during 3-D printing is essential. The widespread use of this emerging technology in industries, along with the lack data on health effects available to consumers in homes and schools, highlights a knowledge gap, which must be filled.

This study sought to evaluate the cellular toxicity of emissions generated from a commercially available 3-D printer, while operating with PC or ABS filaments. Total emissions were collected during printing a 1.5 h print job, which is equivalent to production of a small object. It is probable that not all particles emitted during extrusion of ABS or PC filament were present in the media exposed to cells because of losses in the chamber and the BioSamplers® as well as agglomeration in the media. Chamber wall losses occur, but in our experience with this chamber, the fraction of particles lost is on the order of $10^{-4}$ [92]. Particle losses in the BioSampler® include internal losses (the fraction of particles collected in media that is aerosolized from the collection liquid, adheres to the samplers walls, and remains on the walls), which can be 30% [287] and sampling inefficiency, which may exceed 90% in the 20 to 300 nm size range because small particles have insufficient inertia to be collected in media by the centrifugal collection motion in the sampler [288]. Accounting for chamber and BioSampler® losses, we estimate that less than 25% of emitted particles were collected in the SABM™ media. However, this value is likely underestimated because the number of airborne particles that formed agglomerates in the media after being trapped in media but prior to determination of colloidal concentration is unknown.

This study revealed that the 3-D printer emissions collected in cell culture medium from PC and ABS filaments could be cytotoxic. This observation may be an effect of particle size. It has been well established that smaller particles cause more severe inflammation and oxidative stress than the larger particles at the same administered mass dose due to increased reactive surface area per particle [289, 290]. Note that the size and morphology of particles in culture media that was delivered to cells likely differs from the characteristics of particles emitted into air during 3-D printing. In a prior study, extruding these ABS and PC filaments on the same printer released diffuse clusters of
nanoscale polymer particles with geometric mobility mean sizes of 22.7 nm and 47.5 nm, respectively [285]. In culture media, the measured particle hydrodynamic diameters were about 170 to 200 nm (Supplemental Table 2.2). It is important to clarify that the airborne particle size is the mobility diameter, whereas the particle size in media is the hydrodynamic size. Hence, differences in the basic measurement principle of each technique explain at least some of the observed deviation in particle size. Other factors contributing to the difference in particle size between the aerosol phase and the media include agglomeration in SABM™ if the particle surfaces are hydrophobic. Emitted particles from extrusion of ABS and PC are diffuse clusters with morphology similar to welding fume [126], whereas the particles in the culture medium were denser clusters (Figure S2). Though changes in size and morphology were observed between the aerosol phase and particle in culture media, in humans, inhaled emissions would interact with lung airway-lining fluid and presumably be subject to interactions that alter particle size and morphology as well.

Metals may be present in filaments as constituents of plasticizers. Additionally, to enhance the printed object’s aesthetic, structural, or functional properties, many types of additives and pigments, which may contain transition metals such as Cr, Fe, and Co, are added to the base polymer used to produce 3-D printer filaments. In this study, EDX analysis showed that the main metals of health significance in the ABS and PC particles were Ni, Cr, and Fe. Aside from this study to our knowledge, there is no information on the elemental composition of particles released during 3-D printing with PC filament. The presence of Ni, Cr, and Fe in particles emitted during 3-D printing with ABS were noted in this study as well as in other published studies [90, 115], and might undergo redox-cycling (Fenton or Haber-Weiss reactions) and promote the production of ROS.

The induction of ROS and oxidative stress is one of the central proposed mechanisms of nanoparticle-induced toxicity. ROS are a collective term for the intermediates formed during oxidative metabolism, including both oxygen radicals and non-radical reactive oxygen derivatives. Cells normally maintain a low physiological level of ROS with antioxidant defense systems. Cellular systems are protected from ROS-induced cell injury by an array of defenses composed of various antioxidants with different
functions. When levels of ROS overwhelm defense systems, either due to an increase in ROS production or a decrease antioxidant defense systems, oxidative stress can induce cellular macromolecule damage, including DNA, proteins, and lipids [291]. In this study, we detected elevated production of ROS after exposure to medium containing emissions from printing with PC and ABS filaments. Similar findings were noted by Watson-Wright et al. [292], who reported increased ROS production in SAEC exposed to the particulate matter released from the thermal decomposition of PC thermoplastic.

Oxidative stress is a biological process of an imbalance between the amount of ROS produced and the protective response of antioxidant defense systems. The measurement of antioxidant status can be used indirectly to assess oxidant stress [293]. Most antioxidant enzymes are naturally induced upon ROS production in vivo, and the induction of antioxidant activities decreases or delays ROS-induced oxidative damage. Therefore, the measurement of antioxidant status not only indicates the status of the ROS production but also reflects the propensity of individuals to oxidative damage. In this study, collected PC and ABS emissions decreased TAC in exposed SAEC, indicating oxidative stress.

GPx is an antioxidant enzyme that catalyzes the reduction of hydrogen peroxide (H$_2$O$_2$) to water and lipid peroxides to lipid alcohols via the oxidation of reduced glutathione (GSH) into its disulfide form (GSSH). In the absence of adequate GPx activity or glutathione levels, H$_2$O$_2$ and lipid peroxides are not detoxified and may be converted to OH-radicals and lipid peroxyl radicals, respectively, which may result in oxidative stress-induced pathologies. We found that exposure of SAEC to medium containing PC or ABS emissions decreased total antioxidant capacity and GPx activity. Similarly, Watson-Wright et al. [292] reported that released particulate matter from the thermal decomposition of PC thermoplastic caused a reduction in GSH levels in SAEC. Additionally, exposure to the products of oxidative thermal degradation of ABS caused a decrease of GSH and impairment of membrane integrity in isolated rat hepatocytes [294].

The production of ROS and oxidative stress has been implicated in triggering and modulating apoptosis [295]. In this study, increased apoptosis in SAEC was detected after exposure to PC or ABS emitted-particles collected in cell culture medium, which could be
caused by the impairment of cellular redox homeostasis [296, 297]. Similarly, Watson-Wright et al. [292] demonstrated that the released particulate matter from the thermal decomposition of PC thermoplastic caused a reduction in mitochondrial membrane potential in SAEC, which could suggest a link between the disruption of intracellular redox homeostasis and the stimulation of mitochondrial apoptotic pathway. Additionally, we found that PC and ABS emissions collected in cell culture medium induced necrosis in SAEC. Recent studies found that some components of the apoptotic signaling pathway, such as the BH3-only protein (Bmf), are also crucial in necrotic pathogenesis [298]. Several studies showed that apoptosis and necrosis are closely linked to each other [299, 300]. The apoptotic process may proceed to an autolytic necrotic outcome called secondary necrosis [301].

In an experimental exposure study in healthy human volunteers that investigated acute health effects of 3-D printing using high ultrafine particle-emitting ABS vs. low-emitting polylactic acid (PLA) filaments, after 1 h post inhalation exposure, Gumperlein et al. [273] found no acute effect on inflammatory markers in nasal secretions and urine. In our in vitro study, cellular exposure to medium containing emissions from 3-D printing with PC increased the production of pro-inflammatory cytokines, such as IL-1β, IL-1α, IL-6, IL-12(p70), IL-8, IL-13, IL-16, and TNF-α in SAEC. In contrast, medium containing ABS emissions increased the production of IL-13, IL-8, IL-8, and IL-16. Further, these findings were correlated with increased ROS production and higher occurrence of apoptosis and necrosis in PC emissions-exposed cells. Cytokines and chemokines are known to orchestrate the intricate network of interactions associated with the inflammatory process [302], and play an essential role in the pathogenesis of lung fibrosis, asthma, and chronic obstructive pulmonary disease [303-306]. IL-1 is a central mediator of innate immunity and inflammation and is released from lung epithelial cells to induce inflammatory responses [307]. It was found that IL-1β and TNF-α mediate apoptosis by disrupting normal mitochondrial function in human chondrocytes [308]. Also, IL-1α can be released from cells during apoptosis or necrosis [309-311], alerting the immune system to general tissue damage and initiating a cascade of inflammatory cytokines and chemokines [312, 313]. IL-13 secretion plays a key role in many aspects of airway remodeling [314-316]. Overproduction of IL-13 could be a response to inadequate epithelial repair due to injury.
Previous studies established overexpression of IL-13 by epithelial cells in response to exposure to diesel exhaust using an *in vivo* model [317]. Whether inhalation of 3-D printer emissions triggers inflammatory signaling in humans is still unclear, and further studies using longer exposure times representative of workers are needed.

There are many types of thermoplastic filaments and 3-D printers on the market; it would be a hard task to evaluate all of them in this research study. Therefore, we focused on one printer and two widely used filaments, PC and ABS using a chamber emissions collection system. First, we would like to emphasize that it is not possible to generalize our results from a single printer and two types of filaments to cover all 3-D printing. Second, in this study, major organic compounds present in cell culture medium after 3-D printing were qualitatively identified using SPME/GC-MS. However, it is well known that organic compound emission rates can be on the order of tens to hundreds of micrograms per gram of extruded polymer [93, 95]. Hence, it is possible that not all emitted organic compounds were collected in the cell culture medium during 3-D printing. Third, any reactive chemical species generated may be lost or form new species that may not exist in the printer emissions due to interaction with components of the culture medium. However, the inhaled emissions would interact with lung airway-lining fluid and presumably be subject to interactions as well. It is also possible that if the particles collected in the medium exhibit aqueous solubility, those with greater solubility would be more likely to go into solution or leach adsorbed constituents into the media. Fourth, when inhaled, organic compounds and particles with surface adsorbed organics might not solubilize at the same relative levels as direct exposure to the cells; again, there would be an interaction of deposited contaminants with airway-lining fluid. Fifth, the operation of 3-D printers with polymer feedstock simultaneously releases both particulate and organic compounds, making it difficult to disentangle their relative effects on the observed endpoints. Sixth, the proximity of the BioSamplers® to the printer is only representative of some workplace scenarios where the employee is near a printer, so the amount of emissions collected are likely higher than in a room with ventilation or where an employee only routinely checks the progress of a print job. Finally, even performing repeat print runs of the same filament using the same 3-D printer does not yield exactly the same results each time in terms of number of particles or types and amounts of organic compounds.
Hence, within and between study comparisons of toxicity endpoints will be challenging because of inherent printer variability.

In this study, we observed that the FFF 3-D printer emissions collected in culture medium induced increased production of ROS and expression of pro-inflammatory cytokines and chemokines, as well as apoptosis and necrosis. Based on our results, we believe that the toxicological profiles of PC and ABS emissions are different. However, due to the complexity of these two emissions and different ranges of the particle numbers generated, it is hard to give a conclusion of toxicity between PC and ABS emissions in SAEC at this time. We will address this issue in the future.

2.5. CONCLUSIONS

This study demonstrated that 1) FFF 3-D printing with PC filaments resulted in more particles in cell culture medium than ABS over a similar printing time; 2) exposure to 3-D printer emissions collected in media induced dose-dependent toxicity in SAEC; and 3) emissions collected in media induced ROS production and oxidative stress, increased apoptosis and necrosis, and stimulated secretion of inflammatory chemokines and cytokines. The potential relationship with the observed in vitro effects as well as with hazard and risk of the workplace activity will need further studies with an exhaustive quantitative assessment of the detected chemicals, in vitro testing with defined mixtures of the relevant chemicals.

Conflict of interest

The authors declare no competing financial interest.

Funding

High content screening assays were supported with NTRC funds (921043S) to TS.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

This work has not been reviewed or approved by, and does not necessarily represent the views of, the Commission.
2.8. APPENDIX

Supplemental Figures, Results, and Methods

Artwork / Graphical Abstract

3-D EMISSIONS COLLECTIONS SETUP

Filaments: PC and ABS
Print run: 1.5 h

EMISSIONS CHARACTERIZATION

- Concentration & mean hydrodynamic size (Nanoparticle tracking analysis)
- Zeta potential (Electrophoretic mobility)
- Surface morphology and elemental composition (FE-SEM & EDX)
- Organic Compounds (GC-MS)

CELL TOXICITY EVALUATION

- BKGD.
- PC
- ABS
- 1.5 x 10^4 SAEC / well
- 24 h

- Cell viability
- Cell membrane damage
- Cellular uptake (TEM)
- HCS for ROS production & Apoptosis/ Necrosis
- Total antioxidant capacity
- Glutathione peroxidase
- Cytokines and chemokines

ROS
Apoptosis/ Necrosis
Supplemental Figure 2.1. 3-D emissions collections setup. The FFF 3-D printer emissions were collected in a stainless-steel test chamber.
Supplemental Table 2.1. Average particle concentrations per cm² of the background sample, PC and ABS collected emissions. The particle concentrations were determined using nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern Instruments, Worcestershire, UK). SAEC were seeded in 96-well plates (surface area = 0.32 cm²) and exposed to 100 μL of PC or ABS particles/well collected in cell culture medium.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Dilution, %</th>
<th>Average particles concentration (particles/ml)</th>
<th>Average particles/well</th>
<th>Average particles/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>0</td>
<td>$2.67 \times 10^6$</td>
<td>$2.67 \times 10^5$</td>
<td>$8.34 \times 10^5$</td>
</tr>
<tr>
<td>PC2</td>
<td>0</td>
<td>$3.47 \times 10^7$</td>
<td>$3.47 \times 10^6$</td>
<td>$1.08 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$2.60 \times 10^7$</td>
<td>$2.60 \times 10^6$</td>
<td>$8.13 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>$1.74 \times 10^7$</td>
<td>$1.74 \times 10^6$</td>
<td>$5.42 \times 10^6$</td>
</tr>
<tr>
<td>PC3</td>
<td>0</td>
<td>$6.24 \times 10^7$</td>
<td>$6.24 \times 10^6$</td>
<td>$1.95 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$4.68 \times 10^7$</td>
<td>$4.68 \times 10^6$</td>
<td>$1.46 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>$3.12 \times 10^7$</td>
<td>$3.12 \times 10^6$</td>
<td>$9.75 \times 10^6$</td>
</tr>
<tr>
<td>ABS2</td>
<td>0</td>
<td>$9.08 \times 10^6$</td>
<td>$9.08 \times 10^5$</td>
<td>$2.84 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$6.81 \times 10^6$</td>
<td>$6.81 \times 10^5$</td>
<td>$2.13 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>$4.54 \times 10^6$</td>
<td>$4.54 \times 10^5$</td>
<td>$1.42 \times 10^6$</td>
</tr>
<tr>
<td>ABS3</td>
<td>0</td>
<td>$1.51 \times 10^7$</td>
<td>$1.51 \times 10^6$</td>
<td>$4.72 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>$1.13 \times 10^7$</td>
<td>$1.13 \times 10^6$</td>
<td>$3.54 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>$7.55 \times 10^6$</td>
<td>$7.55 \times 10^5$</td>
<td>$2.36 \times 10^6$</td>
</tr>
</tbody>
</table>
Calculation of estimated particles deposition fraction in the human alveoli based on the International Commission for Radiation Protection (ICRP) reference worker model for particles emitted during FFF 3-D printing with PC and ABS filament

For a print run using ABS that emits particles having a geometric mean diameter of 22.7 nm (GSD = 1.3), modeling using MPPD2 [318] indicates that 35.8% of particles would deposit in the alveoli.

For PC, the geometric mean diameter of airborne particles released during printing is 47.5 nm (GSD = 1.3), and modeling indicates that 23.8% would deposit in the alveoli. Both particle sizes are means from $n = 5$ runs per filament from a prior study [285].


ABS

8 hours work day = 480 min,

20 breaths /min,

1000 ml tidal volume,

- $480 \times 20 \times 1,000 = 9.6 \times 10^6$ total mL breath per work day

Assuming that the average particles concentration in the chamber = $5 \times 10^4$ particles/ cm$^3$ (reasonable based on our previous work [92], the total # of inhaled particles would be

- $5 \times 10^4 \times 9.6 \times 10^6 = 4.8 \times 10^{11}$ particles inhaled

Next, to get the # particles/ cm$^2$ of alveoli

- $(\text{total # inhaled particles}) \times (\text{fraction deposited in alveoli region}) / (\text{total surface area of the lung}) = (4.8 \times 10^{11}) \times 0.358 / (102 \times 10^4) = 1.7 \times 10^5$ particles/ cm$^2$
PC

Same as above but using 0.238 as alveolar deposition fraction = 1.1 x10^5 particles/ cm^2

These calculated values are in good agreement with the in vitro dose values given in Table S2.2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (particles/mL)</th>
<th>Mean Diameter (nm)</th>
<th>Mode Diameter (nm)</th>
<th>D50 (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background^a</td>
<td>0.267 × 10^7 ± 4.06 × 10^5</td>
<td>209.8 ± 13.1</td>
<td>183.9 ± 28.1</td>
<td>172.4 ± 12.6</td>
<td>-19.7 ± 0.7 (pH = 8.0)</td>
</tr>
<tr>
<td>PC1</td>
<td>3.43 × 10^7 ± 1.17 × 10^6</td>
<td>232.6 ± 12.9</td>
<td>163.3 ± 13.0</td>
<td>201.6 ± 13.9</td>
<td>-15.7 ± 2.5 (pH = 7.9)</td>
</tr>
<tr>
<td>PC2</td>
<td>3.47 × 10^7 ± 3.47 × 10^6</td>
<td>201.0 ± 8.0</td>
<td>151.5 ± 8.7</td>
<td>166.8 ± 7.3</td>
<td>-19.1 ± 0.8 (pH = 7.9)</td>
</tr>
<tr>
<td>PC3</td>
<td>6.24 × 10^7 ± 3.66 × 10^6</td>
<td>169.5 ± 8.0</td>
<td>139.5 ± 4.2</td>
<td>147.2 ± 3.1</td>
<td>-19.4 ± 0.3 (pH = 7.9)</td>
</tr>
<tr>
<td>ABS1</td>
<td>2.53 × 10^7 ± 1.22 × 10^6</td>
<td>217.3 ± 1.1</td>
<td>171.1 ± 15.9</td>
<td>196.0 ± 6.5</td>
<td>-21.3 ± 1.8 (pH = 7.8)</td>
</tr>
<tr>
<td>ABS2</td>
<td>0.908 × 10^7 ± 1.39 × 10^6</td>
<td>192.5 ± 9.1</td>
<td>176.3 ± 34.7</td>
<td>173.6 ± 14.3</td>
<td>-17.6 ± 0.6 (pH = 8.0)</td>
</tr>
<tr>
<td>ABS3</td>
<td>1.51 × 10^7 ± 1.47 × 10^6</td>
<td>197.5 ± 9.6</td>
<td>157.8 ± 27.3</td>
<td>170.0 ± 20.1</td>
<td>-18.0 ± 0.7 (pH = 8.0)</td>
</tr>
</tbody>
</table>

^a “Background” refers to chamber air while the printer was powered off and not operating.

Supplemental Table 2.2. Characterization of particles generated during a total of seven print runs (one background, three of PC, and three of ABS): concentration of undiluted collected samples and mean hydrodynamic diameter, mode diameter, and size of the particles in the 50th percentile were measured using NTA, and zeta potential was determined using ELS.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
<th>Compound Name</th>
<th>Compound Structure</th>
<th>% Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>9.833</td>
<td>3.04 x 10⁸ ± 1.11 x 10⁷</td>
<td>Phenol</td>
<td><img src="image" alt="Phenol" /></td>
<td>94%</td>
</tr>
<tr>
<td>PC2</td>
<td>4.65 x 10⁸ ± 8.94 x 10⁷</td>
<td></td>
<td>p-Isopropenylphenol</td>
<td><img src="image" alt="p-Isopropenylphenol" /></td>
<td>94%</td>
</tr>
<tr>
<td>PC3</td>
<td>6.35 x 10⁸ ± 6.41 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>15.942</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>5.04 x 10⁸ ± 7.57 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC3</td>
<td>3.52 x 10⁸ ± 3.13 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>21.533</td>
<td>0.568 x 10⁸ ± 0.155 x 10⁸</td>
<td>Bisphenol A</td>
<td><img src="image" alt="Bisphenol A" /></td>
<td>93%</td>
</tr>
<tr>
<td>PC2</td>
<td>22.00 x 10⁸ ± 1.42 x 10⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC3</td>
<td>13.50 x 10⁸ ± 2.01 x 10⁸</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS1</td>
<td>12.182</td>
<td>0.302 x 10⁸ ± 0.110 x 10⁸</td>
<td>Acetophenone</td>
<td><img src="image" alt="Acetophenone" /></td>
<td>96%</td>
</tr>
<tr>
<td>ABS2</td>
<td>0.61 x 10⁸ ± 6.42 x 10⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS3</td>
<td>0.57 x 10⁸ ± 1.03 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS1</td>
<td>12.797</td>
<td>0.602 x 10⁸ ± 0.717 x 10⁷</td>
<td>α,α-Dimethylbenzenemethanol</td>
<td><img src="image" alt="α,α-Dimethylbenzenemethanol" /></td>
<td>94%</td>
</tr>
<tr>
<td>ABS2</td>
<td>0.71 x 10⁸ ± 6.30 x 10⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS3</td>
<td>0.87 x 10⁸ ± 2.57 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS1</td>
<td>17.103</td>
<td>0.581 x 10⁸ ± 1.88 x 10⁷</td>
<td>Styrene</td>
<td><img src="image" alt="Styrene" /></td>
<td>72%</td>
</tr>
<tr>
<td>ABS2</td>
<td>0.85 x 10⁸ ± 6.45 x 10⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS3</td>
<td>1.10 x 10⁸ ± 2.90 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS1</td>
<td>17.343</td>
<td>0.652 x 10⁸ ± 0.595 x 10⁷</td>
<td>3-Cyclohexen-1-ylbenzene</td>
<td><img src="image" alt="3-Cyclohexen-1-ylbenzene" /></td>
<td>78%</td>
</tr>
<tr>
<td>ABS2</td>
<td>0.45 x 10⁸ ± 3.70 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABS3</td>
<td>0.93 x 10⁸ ± 2.51 x 10⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined

**Supplemental Table 2.3. Volatile organic compounds (VOCs) emitted during a total of seven print runs (one background, three of PC, and three of ABS) identified by GC-MS.**
Supplemental Figure 2.2. Representative chromatograms of the organic compounds emitted during a total of seven print runs (one background, three of PC, and three of ABS). 3-D printer emissions were collected in cell culture medium and organic compounds were analyzed by SPME/GC-MS, followed by tentative identification by comparing mass spectra to the 2014 NIST/EPA/NIH Mass Spectral Library (NIST 14). As we did not quantify the levels of the chemicals, these data should be considered for information only.
Supplemental Figure 2.3. Cytotoxicity of the FFF 3-D printer emissions: cell viability and LDH activity of SAEC exposed to PC (A) and ABS (B) emissions. The PC (prints 2 and 3) and ABS (prints 2 and 3) collected emissions were subjected to vigorous vortex mixing for 5 min, and exposed as 50% dilution, and 25% dilution in serum-free SABM™, and undiluted (0%), resulting in six doses for each filament type (Supplemental Table S1). The background sample was used undiluted. The control samples were treated with SABM™. Following one-way analyses of variance, where the assumptions of the model were examined, Dunnett’s Test was used to determine significance of each treatment compared to either control non-treated cells or background (p<0.05). The symbol (¤) indicate statistically significant differences compared to serum-free cell culture medium treated control (p < 0.05). The number (#) signs indicate statistically significant differences compared to the background (ambient air) treated groups (p < 0.05).
<table>
<thead>
<tr>
<th>Agent</th>
<th>Dilution, %</th>
<th>Average particles/cm²</th>
<th>Analyte, pg/total cells (x 10⁻⁴) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IL-12p70</td>
</tr>
<tr>
<td>Control</td>
<td>NA</td>
<td>0</td>
<td>1.427 ± 0.48</td>
</tr>
<tr>
<td>BKG D.</td>
<td>0</td>
<td>0.834E+06</td>
<td>1.935 ± 0.21</td>
</tr>
<tr>
<td>PC2</td>
<td>50</td>
<td>5.42E+06</td>
<td>2.704 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>8.13E+06</td>
<td>3.980 ± 0.59#</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.8E+06</td>
<td>13.171 ± 0.98#</td>
</tr>
<tr>
<td>PC3</td>
<td>50</td>
<td>9.75E+06</td>
<td>1.445 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>14.6E+06</td>
<td>2.550 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>19.5E+06</td>
<td>3.039 ± 0.22#</td>
</tr>
<tr>
<td>ABS2</td>
<td>50</td>
<td>1.42E+06</td>
<td>1.263 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2.13E+06</td>
<td>1.377 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2.84E+06</td>
<td>1.135 ± 0.07</td>
</tr>
<tr>
<td>ABS3</td>
<td>50</td>
<td>2.36E+06</td>
<td>1.886 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.54E+06</td>
<td>1.642 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4.72E+06</td>
<td>3.228 ± 0.68</td>
</tr>
</tbody>
</table>
Supplemental Table 2.4. Cytokine and chemokine production in SAECs exposed to FFF 3-D printer emissions. SAEC were exposed the PC and ABS emissions in culture medium for 24 h. The PC (print 2 and 3) and ABS (print 2 and 3) collected emissions were subjected to vigorous vortex mixing for 5 min, and exposed as 50% dilution, and 25% dilution in serum-free SABM™, and undiluted (0%), resulting in six doses for each filament type (Supplemental Table S1). The background sample was used undiluted. The control samples were treated with SABM™. Experiments were performed in three independent experiments with n = 3 replicates each. Following one-way analyses of variance, where the assumptions of the model were examined, Dunnett’s Test was used to determine significance of each treatment compared to either control or background (p<0.05). The symbol (¤) indicates statistically significant differences compared to serum-free cell culture medium treated control (p < 0.05). The number (#) signs indicate statistically significant differences compared to the background (ambient air) treated groups (p < 0.05).
CHAPTER 3: PULMONARY AND SYSTEMIC TOXICITY IN RATS FOLLOWING INHALATION EXPOSURE OF 3-D PRINTER EMISSIONS FROM ACRYLONITRILE BUTADIENE STYRENE (ABS) FILAMENT


PMID: 33076715
DOI: 10.1080/08958378.2020.1834034
ABSTRACT
Fused filament fabrication 3-D printing with acrylonitrile butadiene styrene (ABS) filament emits ultrafine particulates (UFPs) and volatile organic compounds (VOCs). However, the toxicological implications of the emissions generated during 3-D printing have not been fully elucidated. The goal of this study was to investigate the in vivo toxicity of ABS-emissions from a commercial desktop 3-D printer. Male Sprague Dawley rats were exposed to a single concentration of ABS-emissions or air for 4 hours/day, 4 days/week for five exposure durations (1, 4, 8, 15, and 30 days). At 24 hours after the last exposure, rats were assessed for pulmonary injury, inflammation, and oxidative stress, as well as systemic toxicity. 3-D printing generated particulate with an average particle mass concentration of 240 ± 90 µg/m³, with an average geometric mean particle mobility diameter of 85 nm (geometric standard deviation = 1.6). The number of macrophages increased significantly at day 15th. In bronchoalveolar lavage, IFN-γ and IL-10 were significantly higher at days 1 and 4, with IL-10 levels reaching a peak at day 15th in ABS-exposed rats. Neither pulmonary oxidative stress responses nor histopathological changes of the lungs and nasal passages were found among the treatments. There was an increase in platelets and monocytes in the circulation at day 15th. Several serum biomarkers of hepatic and kidney functions were significantly higher at day 1. At the current experimental conditions applied, it was concluded that the emissions from ABS filament caused minimal transient pulmonary and systemic toxicity.

Keywords: thermoplastics, emerging technologies, thermal decomposition, printer emissions, printer emitted nanoparticles, volatile organic compounds, inhalation toxicology, pulmonary toxicity, systemic markers.
3.1. INTRODUCTION

Fused filament fabrication (FFF), a three-dimensional (3-D) printing process, is an emerging technology that has recently gained wide popularity among both consumers and manufacturers due to their increased product efficiency, reduced waste, and greater design flexibility. This makes 3-D printing a valuable tool for more sustainable manufacturing and may represent the next generation of manufacturing processes. The projected global market for 3-D printing and related services is expected to grow to almost 50 billion U.S. dollars by 2025 [319], with 6.7 million unit shipments of 3-D printers by 2020. There are many types of 3-D printers used in consumer settings, including those that use solid (filaments) or liquid matrices. The chemical composition and resulting emissions of these types of printers needs further investigation.

FFF 3-D printing involves heating a plastic filament to its melting point and extruding it layer-by-layer to build an object. It has been found that during the thermal decomposition of the filaments, numerous incidental ultrafine particles (UFP) and gaseous or volatile organic compounds (VOCs) with potential adverse respiratory health effects are released into the air [86, 93, 94, 103]. Currently, there are a very limited number of toxicity studies of FFF 3-D printer emissions. In our previous study, we evaluated the toxicity of 3-D printing emissions with ABS filaments (referred to as ABS-emissions thereafter) in an in vitro model using human small airway epithelial cells (SAEC). We found that the mean particle size of the 3-D emissions in cell culture medium was 202 ± 8 nm, and styrene was the predominant VOC. At 24 hours (h) post-exposure, ABS-emissions caused significant dose-dependent cytotoxicity, oxidative stress, apoptosis, necrosis, and production of pro-inflammatory cytokines and chemokines in SAEC cells. Similar findings were reported by another group, which showed that particles in ABS-emissions from a consumer-level 3-D printer caused decreased cell viability and increased oxidative stress and inflammatory responses. Stefaniak et al. [223] investigated the acute toxic effects of ABS-emissions on cardiovascular function via nose-only inhalation study in rats. Exposure to 1 mg/m³ ABS-emission for 3 h induced significantly higher mean arterial pressure concomitant with elevated resting arteriolar tone and impaired endothelium-dependent arteriolar dilation. Their results indicated that
the 3-D printer emissions could potentially induce systemic toxic effects, similar to what was described previously in relation to inhalation of nanoparticles and ultrafine particulate (UFPs) matter [277, 278]. In a health survey, about 60% of participants using 3-D printing in commercial prototyping businesses, educational institutions, and public libraries reported weekly respiratory symptoms [267]. The same study also determined that working more than 40 h per week was significantly associated with asthma or allergic rhinitis diagnosis. In a randomized, cross-over design study, healthy human volunteers were exposed to ABS 3-D printer emissions for 1 h [273]. No acute effects on inflammatory markers in nasal secretions or urine were found. However, a slight increase in exhaled nitric oxide was noted, which could be induced by eosinophilic inflammation from inhaled UFPs. In a case report study [268], a self-employed businessman with a history of childhood asthma operated ten 3-D printers with ABS filaments in a small work area, and, after 10 days working with ABS printing, he experienced chest tightness, shortness of breath, and coughing.

Given that the use of this technology has expanded from workplaces/industry or small businesses to schools and homes and the lack of data addressing the toxicity of emissions, there is a critical need to fill knowledge gaps and assess the potential toxicological effects of exposure to the FFF 3-D emissions, which would help to establish effective control strategies and exposure limits for specific materials to prevent adverse health effects from 3-D printing emission exposure. This study sought to evaluate the in vivo respiratory and systemic toxicity of emissions from FFF 3-D printing with ABS filaments. A real-time 3-D printer generation system was designed to allow for simultaneous printing of three commercially available desktop 3-D printers and delivery of an aerosol comprised of a mixture of particles and VOCs to the animal exposure chamber. A time-course exposure study was conducted via whole-body inhalation exposure. Male Sprague-Dawley rats were exposed to a single concentration (240 µg/m³ ABS average particle mass concentration) for 4 h/day throughout five exposure durations: 1, 4, 8, 15, and 30 days (4 days/week). At 24 h after the last exposure, histopathological changes in the nasal cavity tissue and pulmonary injury, inflammation, and fibrotic responses, as well as systemic toxicity blood markers, were assessed.
3.2. MATERIALS AND METHODS

3.2.1. Three-dimensional printer emissions inhalation exposure system

An inhalation exposure system was specifically designed and constructed to deliver FFF 3-D printer emissions to a whole-body rodent exposure chamber (Figure 3.1). The airtight 55 cm × 55 cm × 50 cm (L × W × H) exposure chamber was constructed out of 16-gauge stainless steel with a clear polycarbonate door. The system automatically controlled chamber pressure, airflow, temperature, particle concentration, and exposure time using custom software. During exposures, rats were housed in a stainless-steel cage rack, which could hold up to 12 rats in individual cage partitions. The cage rack rested on top of cage support beams, which were 1 cm outside diameter stainless steel tubes with small holes (0.33-cm diameter) drilled into the undersides. Each hole was placed at the center of each cage partition such that aerosols would be drawn to each animal's breathing space.

The exposure chamber discharged exhaust air into a carbon/HEPA filter bank. The flow rate of the exhaust was controlled by a mass flow controller (MCRW-50-DS; Alicat Scientific, Tucson AZ) that had its downstream port connected to a vacuum. During exposures, this flow was held constant at 30 l/min. The air entered the top of the exposure chamber either from being pulled through an airtight chamber (50 cm × 64 cm × 122 cm L × W × H) that housed 3 consumer-grade desktop FFF 3-D printers (Manufacturer A) or from a mass flow controller (MCR-50, Alicat Scientific) that provided filtered dilution air.

The exposure chamber pressure was monitored using a differential pressure transducer (Model 264; Setra Systems, Inc. Boxborough MA). Under typical exposure conditions, filtered air was set to zero flow, and the pressure inside the exposure chamber was -6.35 cm H₂O to ambient. This slight negative pressure pulled air from the 3-D printer housing chamber into the exposure chamber via conductive silicone tubing (0.80 cm ID). Room air entering the 3-D printer housing chamber first passed through a HEPA filter and carbon bed.

The temperature and relative humidity inside the exposure chamber were continuously monitored using an electronic probe (HMP60; Vaisala Corporation, Helsinki
Finland). The 3-D printers produced significant heat during operation. Therefore, the inhalation exposure chamber was fitted with a cooling device to maintain air temperatures between 22 and 24 °C. This was necessary for animal welfare and was more representative of the real-world exposure condition where room air helps cool down the heat emission in larger spaces. A thermoelectric module (CP-110; TE Technology, Inc., Traverse City, MI) was fitted to the outside back wall of the exposure chamber using four mounting holes. Inside the exposure chamber, custom cooling fins were mounted through the same four mounting holes directly onto the other side of the thermoelectric module. These cooling fins were made of 6061 aluminum and had two circulating fans. The exposure system’s custom software monitored the exposure chamber’s air temperature and activated the cooler as needed. The two circulating fans always remained active during operation.

3.2.2. Three-dimensional printer settings for ABS filament

The three FFF 3-D printers were each programmed to print simultaneously an object shown in Supplemental Figure 3.1. from a commercially available ABS filament. The objects printed were 12.7 cm wide by 12.7 cm long, with a height of about 2.54 cm. The width and length were chosen because it occupied most of the build plate. Preliminary testing showed that this type of object produces more particles than a part with a smaller footprint. The object used 240 g of filament to fully print. The 3-D printers all used a nozzle size of 0.4 mm. When not in use, the filament was stored at room temperature in an airtight dry box. Supplemental Table 3.1. details the 3-D printer settings. These settings produced 3-D printed parts without layer separation, warping, and a smooth outer surface.
Figure 3.1. Diagram of the custom-built computer automated inhalation exposure system for testing the ABS 3-D printing emissions. The exposure chamber air was exhausted into a carbon/high-efficiency particulate air (HEPA) filter bank. The flow rate of this air was controlled by a mass flow controller that had its downstream port connected to a hose vacuum. During exposures, this flow was held constant at 30 l/min (LPM). The airflow was controlled using multiple mass flow controllers (MFC) operated at continuous flow rates. Animals were exposed to fumes (particles and gasses) generated from three commercially available 3-D printers placed inside an airtight chamber (300 l volume).
3.2.3. ABS emissions collection and characterization

3.2.3.1. Particle collection and characterization

The aerosol mass concentration inside the exposure chamber was continuously monitored with a Data RAM (DR-40000; Thermo Electron Co., Waltham, MA), and gravimetric determinations (37 mm cassettes with 0.45 µm pore-size Teflon filters, 2 l/min sample flow) were used to calibrate and verify the Data RAM readings during each exposure run. For this study, a 4-h average concentration of 250 µg/m³ was delivered. Particle counts were also recorded by the custom software connected to a condensation particle counter (CPC) (Model 3787; TSI Inc. Shoreview, MN).

A fast mobility particle sizer (FMPS, Model 3091, TSI Inc.) was used to collect particle size data in 5-second intervals during several 4-h test exposures. The test exposures were conducted in the same manner as a typical exposure run, but without animals present in the exposure chamber. In addition, during these test runs, a field emission – scanning electron microscope (FE-SEM; Hitachi S-4800, Tokyo, Japan) was used to analyze particle physical morphology by drawing aerosol samples, for 20 min at a flow rate of 1 l/min, from the exposure chamber onto 25 mm 0.1 µm pore sized polycarbonate filters.

3.2.3.2. VOCs collection and characterization

VOCs in the ABS-emissions were sampled and analyzed according to the NIOSH Manual of Analytical Method (NMAM) 3900 (NIOSH 2018). The emissions were sampled using fused-silica lined, evacuated canisters (450 ml; Entech Instruments Inc., Simi Valley, CA) with a 3-h capillary flow controller and analyzed for a suite of VOCs using an Entech 7200/7650 preconcentration system coupled with an Agilent 7890/5975 gas chromatograph/mass spectrometer (GC/MS) (Santa Clara, CA). These samples were taken from the exposure chamber during the first 4-h of test runs without animals present. The VOC collection was repeated on five different days, and the average and standard deviation were calculated for each VOC identified.
3.2.4. Animals

Male Sprague-Dawley [Hla: (SD) CVF] (SD) rats (6-7 weeks old, 200–225 g) were purchased from Hilltop Lab Animals (Scottdale, PA), housed in ventilated polycarbonate cages, and acclimated for at least seven days before the study began. The animals were provided HEPA-filtered air, irradiated Teklad 2918 diet (Harlan, Madison WI), a combination of ALPHA-dri® and Teklad sani-chips as bedding and tap water ad libitum. Rats were housed four per cage with a 12-h light-dark cycle, and the facility was maintained at 22 ± 2 °C and 40–60% humidity. The study protocol was reviewed and approved by the CDC-Morgantown Institutional Animal Care and Use Committee. The CDC-Morgantown Institutional Animal Care and Use Committee is accredited by AAALAC International.

3.2.5. Experimental design

After acclimatization, the animals were randomly divided into two groups (n = 8 per group) and exposed by whole-body inhalation to HEPA- and carbon-filtered air or an average concentration of 240 µg/m³ ABS-emissions, 4 h/day, 4 days/week for 1, 4, 8, 15, and 30 day exposure durations. (Additional information regarding the animal exposure paradigm can be found in the Supplemental material). At 24 h after the last exposure, the rats were euthanized following an intraperitoneal injection of 100-200 mg sodium pentobarbital/kg body weight (Fort Dodge Animal Health; Fort Dodge, IA). Whole blood was collected from the abdominal aorta and transferred to two collection tubes: a vacutainer containing EDTA (used for whole blood hematological analysis), and a vacutainer containing clot activator and a polymer gel (used for serum chemistry analysis) (Becton-Dickinson; Franklin Lakes, NJ). After clamping the left lung lobe and tying off the right cardiac lobe, bronchoalveolar lavage fluid (BALF) was collected from the remaining right lobes (BALF collection further described below). After lavage, the tied-off right lung lobe and the lavaged right lobes were collected and stored at -80 °C. The left lung lobe and the head/nasal tissues were preserved for histopathological evaluation (Representation of experimental design is displayed in Supplemental Figure 3.2).
3.2.6. Particle deposition estimates in the nasal passages, tracheobronchial, and alveolar regions

The ABS-emissions particle deposition mass for the head/nose region, tracheobronchial region, alveolar region, and the sum of the tracheobronchial and alveolar regions were estimated using the MPPD model [320] based on two different scenarios: without clearance and with clearance, respectively. Furthermore, the in vivo alveolar deposition estimates were compared to our previous in vitro SAEC study results (Supplemental material).

3.2.6.1. Particle deposition mass without clearance

Particle mass deposition in nasal passages and lung regions was calculated. The size distribution data (particle count based) provided by the FMPS of the ABS-emissions inside the exposure chamber was converted to mass distribution. For this conversion, all particles were assumed spherical with a density of 1.04 g/cm³ (density of the ABS filament). The FMPS instrument software was used for the calculations. This estimated mass distribution was used as input into the MPPD model [320] with the following parameters: animal breathing rate of 120 breaths/min, tidal volume of 1.7 ml, 4-h of total exposure time, and 240 µg/m³ as the 4-h average concentration.

3.2.6.2. Particle deposition mass with clearance

Exposures were conducted for four consecutive days, followed by three rest days. In order to account for clearance, deposition in the nose, tracheobronchial and alveolar regions calculated in the model without clearance were used in a discrete-time model that accounted for clearance from each region between the daily exposures. The particle mass deposited for a day of exposure was taken from the one-day exposure particle mass depositions listed in the model without the clearance described above. The deposition for each region was assumed to be the same each subsequent day of exposure. On each day, the amount of material remaining in each region was taken as the sum of the amount present in that region from the previous day, plus the one-day exposure particle mass deposition, minus one-day clearance for each region. The one-day clearance percentages were 100% for the nose, 90% for the tracheobronchial region, and 4% for
the alveolar region. This was repeated for the first four-day exposure. For the next three rest days (Friday, Saturday, and Sunday), only clearance from the various compartments was assumed. The process of 4 day exposures and 3 rest days with daily clearance patterns was repeated for the remaining exposure duration groups (8, 15, and 30 days).

3.2.7. Bronchoalveolar lavage analysis

3.2.7.1. Bronchoalveolar lavage fluid collection and cytology

After exsanguination, the trachea was cannulated, the chest cavity opened, and BALF collected from the right lung (except the cardiac lobe) via the tracheal cannula. The first BAL fraction was obtained by gently filling the right lung with 6 ml cold PBS, massaging for 30 sec, withdrawing, and repeating the process for another 30 sec. The second BAL fraction consisted of subsequent aliquots of 5 ml PBS (instilled and withdrawn) that were combined until a 15 ml volume was obtained. Both BAL fractions were centrifuged (800 × g for 10 min at 4 °C). The supernatant from the first fraction was set aside to measure total protein, lactate dehydrogenase activity, as well as surfactant and cytokine levels. After discarding the supernatant from the second BAL fraction, the cell pellets from both BAL fractions were combined, resuspended in 1 ml of PBS (Lonza, Pearland, Texas), and aliquots were used to determine the total cell number and cell differential. Briefly, total cell counts were quantified using a Beckman Coulter Multisizer 4 particle counter (Coulter Electronics; Hialeah, FL), and morphology identified using cytospin preparations stained with HEMA solutions (Fisher Scientific; Kalamazoo, MI), counting at least 500 cells per slide. The numbers of alveolar macrophages (AMs), polymorphonuclear leukocytes (PMNs), lymphocytes, and eosinophils were normalized to the total number of cells.

3.2.7.2. Transmission electron microscopy (TEM) staining of BAL cells

BAL cells were fixated in 1 ml Karnovsky’s fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M sodium cacodylic buffer) and stored at 4 °C until processing. The cells were post-fixed in 2% osmium tetroxide for 1 h, mordanted in 1% tannic acid, and stained en bloc in 0.5% uranyl acetate. After a graded series of ethanols for dehydration, the samples were infiltrated in propylene oxide. The samples were
embedded in EPON™ (epoxy resin). The blocks were sectioned at 70 nm, and the resulting grids were stained with 4% uranyl acetate and Reynold's lead citrate. The sections were imaged on a JEOL 1400 transmission electron microscope (JEOL, Tokyo, Japan).

3.2.7.3. **Scanning electron microscopy (SEM) images of lungs**

A 5-micron non-lavaged lung paraffin section was mounted on a carbon planchet and deparaffinized with propylene oxide for 30 min. The sample was sputter-coated with gold-palladium for 30 sec and imaged using a Hitachi S4800 field emission scanning electron microscope (Tokyo, Japan) at 5 kV.

3.2.7.4. **Total protein and LDH activity**

Total protein levels and lactate dehydrogenase (LDH) activity were evaluated from the acellular first BAL fraction by using the Pierce™ BCA Protein Assay Kit (Fisher Scientific) and Lactate Dehydrogenase Reagent Set (Pointe Scientific; Lincoln Park, MI), respectively. Data were acquired using Synergy H1 Microplate Reader (BioTek; Winooski, VT).

3.2.7.5. **Surfactant proteins A and D**

Surfactant protein A and D, known to contribute to surfactant homeostasis and pulmonary immunity, were quantified from the 3-fold diluted first BAL fractions using surfactant associated protein A (SP-A) and surfactant associated protein D (SP-D) kits (Biomatik USA; Wilmington, DE), according to the manufacturer guidelines. Data were acquired using Synergy H1 Microplate Reader (BioTek, Winooski, VT).

3.2.7.6. **BALF cytokines levels**

The levels of pro- and anti-inflammatory cytokines were measured from undiluted first BAL fraction, *i.e.*, interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-10, IL-13, interferon (IFN)-gamma, neutrophil-activating protein 3 (KC/GRO), and tumor necrosis factor (TNF)-alpha were measured from undiluted first BAL fractions using a V-PLEX Pro-inflammatory Panel
2 Rat Kit (MSD; Meso Scale Discovery, Rockville, MD), according to the manufacturer protocol. Data were acquired using a QuickPlex SQ 120 plate reader (MSD).

### 3.2.8. Oxidative stress markers

The right lung (except the cardiac lobe) was homogenized with a Bead Mill 24 Homogenizer (Fisher Scientific International, Inc.; Hampton, NH) for 2 min at 4 °C in 1 ml cold PBS (pH 7.4) containing protease inhibitor cocktails and EDTA (Halt™ Protease Inhibitor Cocktails, Thermo Scientific™, Waltham, MA). The protein carbonyl and the malondialdehyde (MDA) levels were quantified from 100-fold diluted lung homogenate suspensions using Protein Carbonyl content and Lipid Peroxidation assay kits (Cell Biolabs, Inc.; San Diego, CA), respectively, according to the manufacturer's protocol. Data were acquired using a Synergy H1 Microplate Reader (BioTek, Winooski, VT). The concentrations were normalized to the total protein content determined using the Pierce™ BCA Protein Assay Kit (Fisher Scientific International, Inc., Hampton, NH).

### 3.2.9. Blood processing and analysis

Whole blood collected from each rat was equally divided between a vacutainer tube containing EDTA as an anticoagulant (hematological analysis) and a tube containing spray-coated silica, to help in clotting, and a polymer gel (serum separation and analysis).

#### 3.2.9.1. Characterization of blood cells and hematological parameters

Complete blood count (CBC) tests were performed 30-45 minutes post-exposure to evaluate hematological parameters, which included peripheral erythrocyte and leukocyte counts, leukocyte differentials (percent lymphocytes, neutrophils, monocytes, basophils, and eosinophils), platelet counts, mean platelet volume (MPV), hemoglobin levels, hematocrit, mean corpuscular hemoglobin (MCH) and hemoglobin concentration (MCHC), red blood cell distribution width (RDV), reticulocyte counts, mean platelet volume (MCV), and platelet distribution width (PDW), using a ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Inc., Westbrook, ME).
3.2.9.2. Serum chemistry profile

For serum chemistry analysis, the whole blood was allowed to clot at room temperature and centrifuged at 2,500 rpm for 10 min. Using a Catalyst One Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME), the following serum biochemical parameters were evaluated: albumin (ALB), globulin (GLOB), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatine kinase (CK), creatinine (CREA), C-reactive protein (CRP), lactate dehydrogenase (LDH), ammonia (NH₃), inorganic phosphate (PHOS), total bilirubin (TBIL), total protein (TP), triglycerides (TRI), and uric acid (URIC).

3.2.9.3. Serum cytokine levels

The levels of pro- and anti-inflammatory cytokines IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-gamma, KC/GRO, and TNF-α, were determined from samples diluted 4-fold using the V-PLEX Pro-inflammatory Panel 2 Rat Kit (MSD), according to the manufacturer protocol. Data were acquired using a QuickPlex SQ 120 plate reader (MSD).

3.2.9.4. Serum immunoglobulin E

The quantity of immunoglobulin E (IgE) was measured from 2-fold diluted samples using a Rat IgE ELISA Kit (Abcam; Cambridge, MA), according to the manufacturer's protocol. Data were acquired using a Synergy H1 Microplate Reader (BioTek).

3.2.10. Lung and nasal passages histopathological evaluation

The left lung (non-lavaged) was inflated with 10% neutral buffered formalin (NBF), embedded in paraffin, cut at 5 microns on Schott slides, and stained with Hematoxylin and Eosin (H&E) for histopathological evaluation. Lesions were reviewed by a board-certified veterinary pathologist and classified as following: within normal limits (WNL); 1, minimal (change barely exceeds that which is considered normal); 2, mild/slight (the lesion is easily identified but is of limited severity); 3, moderate (the lesion is prominent, but there is significant potential for increased severity); 4, severe (the change is as complete as possible, occupies the majority of the organ).
After the lungs were processed, the nasopharynx was lightly flushed with 10% NBF, and the nasal passages were collected. Nasal tissues were fixed in formalin for approximately one week, then decalcified in 13% formic acid. Standard nasal sections (T1, T2, T3, and T4) were taken [321]. The sections were embedded in paraffin, cut at 5-microns, and stained with H&E.

3.2.11. Statistical analysis

All statistical analyses were performed using SAS/STAT for Windows v9.4. Two-way analyses of variance (treatment by duration) were performed on the measured variables. Post-hoc comparisons between relevant groups were performed using Fishers LSD. All analyses are considered significant at p < 0.05.

3.3. RESULTS

3.3.1. ABS printing emissions characterization

3.3.1.1. Particle mass and count concentrations

Chamber particle concentration readings during four representative exposure runs are shown in Figure 3.2. All runs shown had the same 4-h mass average of 240 µg/m³. In order to ensure that the same 4-h average particle mass was delivered daily at a constant and repeatable exposure dose, the exposure system software calculated every two sec what the 4-h average mass concentration would be if particles were immediately not delivered to the exposure chamber. If the mass concentration exceeded 240 µg/m³, the system automatically set the mass flow controller for the dilution flow to 30 l/min, thus providing the exposure chamber with filtered air rather than air from the 3-D printer housing chamber. This method of average particle concentration control was used because significant inconsistencies were observed, day-to-day, in particle generation from the 3-D printers even though they were all ABS printers from a single manufacturer.
Figure 3.2. Chamber particle characterization. A) Particle mass concentrations (red lines), and particle counts (blue lines) over time; B) Particle size distribution plot count-based; C) Particle size distribution from count based converted to mass by assuming all particles were spherical and had a density of 1 g/cm³; D) Particle geometric mean electric mobility diameter during two 4-h print jobs.
The average mass concentration, determined with gravimetric filters, was 240 \( \mu g/m^3 \) with a standard deviation of 90 \( \mu g/m^3 \), and the daily average count concentration measured by the CPC was 88,400 particles/cm\(^3\) with a standard deviation of 23,000 particles/cm\(^3\). Typical particle count concentrations peaked during the first 10 min of printing at about a million particles per cm\(^3\), and from 20 min to 120 min, the count concentration slowly declined. From 120 min to the end of the print job, the particle count gradually increased. Particle mass readings peaked between 40 and 60 min into the print job, then slowly decreased either through the remaining print job or had a second peak mid-print (120 min).

3.3.1.2. Particle size

A typical size distribution plot (particle count based) of the ABS-emissions inside the exposure chamber during a test run is shown in Figure 3.2B. This type of data was collected every 5 secs using the FMPS. Figure 3.2C shows the data from Figure 2B converted to mass by assuming all particles were spherical and had a density of 1 g/cm\(^3\). The geometric mean diameter (electric-mobility-based) for every 5-second sample during two separate 4-h test runs is shown in Figure 3.2D. The geometric mean diameter during the start of the print jobs was 40 nm, and it increased to approximately 85 nm after 20 min and remained between 79 nm to 95 nm for the remainder of the 4-h print. The geometric standard deviation was 1.6.

The particles were also sampled onto filters and imaged with a scanning electron microscope (SEM). Several representative particles are shown in Figure 3.3. The circular dark holes are the pores in the polycarbonate filter. The typical physical diameters of the particles ranged from 40 nm to 500 nm. Some of the particles were elongated and not perfectly spherical with rounded edges.
Figure 3.3. Representative images of ABS 3-D printer-emitted particles indicating surface morphology. 3-D printer emissions were analyzed using FE-SEM. Scale bar at 500 nm for all images.
3.3.1.3. Analysis of volatile organic compounds by GC/MS

Table 3.1 lists the most commonly detected volatile compounds (VOCs) in the emissions from five ABS print runs. The emissions were analyzed by GC/MS over a 4-h collection time. Included in Table 3.1 are the OSHA and NIOSH permissible exposure limits (PEL) and recommended exposure limits (REL) for these VOCs, respectively, for an 8-h work shift. The levels of benzene and acetaldehyde were the highest among the detected compounds, though much lower than the PEL and REL.

3.3.2. Particle deposition estimates in the nasal passages, tracheobronchial, and alveolar regions

3.3.2.1. Particle deposition mass without clearance

The particle deposition estimates for the nasal passages, the tracheobronchial, and the alveolar regions are provided in Table 3.2.

3.3.2.2. Particle deposition mass with clearance

The estimated particle mass burden remaining in each region after the specified number of days of exposure is given in Table 3.3.
Table 3.1. VOCs in the ABS-emissions were sampled and analyzed according to the NIOSH Manual of Analytical Methods (NMAM) 3900 (NIOSH 2018).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average (ppm)</th>
<th>Standard deviation</th>
<th>OSHA PEL (ppm)</th>
<th>NIOSH REL (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Hexanedione</td>
<td>0.0011</td>
<td>0.0022</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.1105</td>
<td>0.0331</td>
<td>200</td>
<td>None</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.0112</td>
<td>0.0109</td>
<td>1000</td>
<td>250</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.0045</td>
<td>0.0037</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.0036</td>
<td>0.0014</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>D-Limonene</td>
<td>0.003</td>
<td>0.0015</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.019</td>
<td>0.0106</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Isopropyl Alcohol</td>
<td>0.0034</td>
<td>0.0067</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Methyl Methacrylate</td>
<td>0.0037</td>
<td>0.003</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Styrene</td>
<td>0.0024</td>
<td>0.003</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.0084</td>
<td>0.0168</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>m,p-Xylene</td>
<td>0.0024</td>
<td>0.0005</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Days of exposure</td>
<td>Particle mass deposited (µg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Nose</td>
<td>Tracheobronchial</td>
<td>Alveolar</td>
<td>Total Lung</td>
</tr>
<tr>
<td>1</td>
<td>0.54</td>
<td>0.23</td>
<td>0.85</td>
<td>1.08</td>
</tr>
<tr>
<td>4</td>
<td>2.16</td>
<td>0.91</td>
<td>3.41</td>
<td>4.32</td>
</tr>
<tr>
<td>8</td>
<td>4.32</td>
<td>1.82</td>
<td>6.81</td>
<td>8.63</td>
</tr>
<tr>
<td>15</td>
<td>8.09</td>
<td>3.42</td>
<td>12.77</td>
<td>16.19</td>
</tr>
<tr>
<td>30</td>
<td>16.19</td>
<td>6.83</td>
<td>25.54</td>
<td>32.38</td>
</tr>
</tbody>
</table>

**Table 3.2. Modeled total lung burden without clearance.**

<table>
<thead>
<tr>
<th>Days of exposure</th>
<th>Particle mass remaining at the end of exposure (µg)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nose</td>
<td>Tracheobronchial</td>
<td>Alveolar</td>
<td>Total Lung</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.2</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.3</td>
<td>3.2</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>0.3</td>
<td>5.9</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
<td>0.3</td>
<td>9.2</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>0.3</td>
<td>12.1</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3. Modeled lung burden estimates with clearance.**
3.3.3. Pulmonary Injury and Inflammation

Figure 3.4 represents SEM images of lungs from animals exposed to ABS-emissions, confirming that the ABS particle reached and deposited in the alveolar region as early as after one day of exposure.

A TEM analysis of BAL cells was carried out to determine the cellular uptake of particles and morphological changes. Figure 3.5 revealed the pulmonary ABS particle uptake by alveolar macrophages in BAL cells after one and 30 days of exposure (similar outcomes were observed at all durations of exposure). The images showed particle uptake in membrane-lined vacuoles or particle localization in the cytoplasm (arrows). Gross analysis of the morphological appearance of BAL cells indicated no significant differences in cellular morphology.

For the assessment of pulmonary damage, total lung cells, and differentiated cells were counted in the BALF (Figure 3.6). The number of macrophages was significantly higher (33%) only at day 15 of exposure. Exposure to ABS emissions caused slightly increased total cell numbers in BALF from day 1 to day 15 of exposure. However, this increase was not significant when compared to air-control rats, and by the 30th day of exposure, total cell numbers returned to control levels. Similarly, there were no significant differences in BALF neutrophil, lymphocyte, and eosinophil counts at any exposure duration, although the numbers of neutrophils and lymphocytes were slightly increased in ABS-exposed rats throughout the entire study.
Figure 3.4. Representative images of ABS particles deposited in the alveolar region at days 1 and 30 of exposure using FE-SEM: (A) air-control, (B, C, D, E) day 1 of exposure, (F, G, H, I) day 30 of exposure. Scale bar at 10 µm for images B, D, and F, 5 µm for image H, 1 µm for images C, E, and G, and 500 nm for image I.
Figure 3.5. Cellular uptake of ABS particles in BAL cells at days 1 and 30 of exposure by TEM: (A) air-control, (B, C) day 1 of exposure, (D, E) day 30 of exposure. Scale bar at 1 µm for images A, B, and D, and 500 nm for images C and E.
Figure 3.6. BAL cell differential. Total cells (A), AMs (B), PMNs (C), lymphocytes (D), and eosinophils (E). The rats were exposed for 1, 4, 8, 15, and 30 days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represent means ± SEMs; N = 8/group/time point. *Significantly different from corresponding air control, p < 0.05.
To further examine the pulmonary injury, LDH activity, total protein, SP-A, and SP-D levels in BALF were measured. We found that the LDH activity and total protein levels were not significantly different between the ABS-exposed animals and the air-control group at any time point (Figure 3.7). There were also no significant differences in SP-A and SP-D in the BALF (Figure 3.8). However, the levels of these two proteins, known for their protective role in lung disease, were slightly decreased in ABS-exposed rats, which suggests that the emissions might have the potential to alter the host’s lung immune responses. Specifically, SP-A levels decreased beginning at the 8th day of exposure and persisted until day 30; SP-D levels decreased starting with the 4th day of exposure and lasted until the 15th day of exposure.

To evaluate ABS emission-induced inflammatory responses in airways, a panel of 9 pro- and anti-inflammatory cytokines were measured in BALF. Significant changes were observed in BALF IFN-γ and IL-10 levels, with no significant differences in levels of IL-1β, IL-4, IL-5, IL-6, KC, and TNF-α when compared to air-control rats (Table 3.4). IFN-γ levels increased early (73% at day 1) and returned to control levels after day 4. Similarly, IL-10 levels significantly increased early, but remained elevated, peaking at day 15 day, a 39% increase compared to air-control animals.

Along with inflammation, oxidative stress is a key mechanism linking particle exposure to observable physiological and biochemical responses. As such, protein carbonyl and MDA levels were measured to determine the extent of protein and lipid oxidation after exposure to ABS emissions (Figure 3.9). There were no significant differences in protein carbonyl and MDA levels between exposed and air-control rats at any exposure time point. However, similar to surfactant protein levels, protein carbonyl concentrations were slightly increased in ABS-exposed rats from day 1 through day 15, returning to control values by day 30.
Figure 3.7. Pulmonary injury markers: LDH activity (A) and total protein (B) in BALF. The rats were exposed for 1, 4, 8, 15, and 30 days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represents means ± SEMs; N = 8/group/time point.

Figure 3.8. Markers of injury to the alveolar epithelium: surfactant proteins SP-A (A) and SP-D (B). The rats were exposed for 1, 4, 8, 15, and 30-days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represents means ± SEMs; N = 8/group/time point.
Figure 3.9. Lung oxidative stress markers: protein carbonyls (A) and malondialdehyde (B). The rats were exposed for 1, 4, 8, 15, and 30 days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represents means ± SEMs; N = 8/group/time point.
3.3.4. Systemic effects

To assess for exposure-induced systemic toxic effects after pulmonary exposure to ABS emissions, blood samples were analyzed for clinical and hematological parameters, in addition to biomarkers of muscle, metabolic, renal, and hepatic function. Significant increases in platelet (2-fold) and monocyte (73%) levels were measured at day 15 of exposure (Table 3.5), accompanied by a nonsignificant increase in platelet and monocyte populations on day 30 (62% and 39%, respectively) when compared to air-control. There were no significant differences in the other hematological measurements at any time point, although elevations were observed for neutrophils and eosinophils (35% and 53%, respectively), peaking on day 15. Serum chemistry analysis indicates that creatinine kinase and aspartate aminotransferase, markers of hepatic and kidney function, were significantly higher after one day of exposure, returning to control levels by day 4 (Table 3.6). In addition, although the levels of inorganic phosphate and lactate dehydrogenase, markers of hepatic function were within normal intervals, they were significantly higher in ABS-exposed animals when compared to air-controls. Similarly, after 15 days of exposure, blood urea nitrogen, inorganic phosphate, and uric acid were significantly higher, with concentrations falling within standard ranges. No significant changes in any of the serum chemistry markers were observed after 30 days of exposure.

The systemic inflammation was further assessed by measuring the response of pro- and anti-inflammatory cytokines (similar panel as assessed in BALF) in serum. Furthermore, there were no significant differences in serum levels of pro- or anti-inflammatory cytokines, at any time point (Supplemental Table 3.2). Slightly higher serum levels of IFN-γ, IL-6, IL-10, and KC were observed at day 15. IFN-γ, IL-6, and IL-10 remained slightly increased at exposure day 30, as well as serum levels of IL-1β.

No significant difference in serum IgE levels, an immune-mediated marker of allergic response, was observed (Supplemental Figure 3.4).
Table 3.4. Level of cytokines in bronchoalveolar lavage fluid. Values represents means ± SEMs; n = 8/group/time point. *p<0.05 versus air-control group.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Exposure duration (days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Air</td>
<td>4.86 ± 0.58</td>
<td>6.64 ± 0.23</td>
<td>7.27 ± 0.17</td>
<td>7.25 ± 0.50</td>
<td>7.07 ± 0.42</td>
</tr>
<tr>
<td>(M/μL)</td>
<td>ABS</td>
<td>5.15 ± 0.42</td>
<td>6.76 ± 0.15</td>
<td>6.90 ± 0.30</td>
<td>7.43 ± 0.32</td>
<td>7.01 ± 0.97</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Air</td>
<td>10.05 ± 1.20</td>
<td>13.06 ± 0.39</td>
<td>13.79 ± 0.18</td>
<td>13.15 ± 0.86</td>
<td>12.09 ± 0.68</td>
</tr>
<tr>
<td>(g/dL)</td>
<td>ABS</td>
<td>10.54 ± 0.80</td>
<td>13.18 ± 0.18</td>
<td>12.91 ± 0.56</td>
<td>13.58 ± 0.54</td>
<td>12.05 ± 1.68</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>Air</td>
<td>32.23 ± 2.27</td>
<td>38.57 ± 1.22</td>
<td>40.40 ± 0.94</td>
<td>37.61 ± 2.74</td>
<td>34.93 ± 2.27</td>
</tr>
<tr>
<td>(%)</td>
<td>ABS</td>
<td>30.43 ± 2.63</td>
<td>39.11 ± 0.43</td>
<td>37.84 ± 1.88</td>
<td>39.33 ± 1.87</td>
<td>34.95 ± 4.86</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>Air</td>
<td>59.64 ± 0.85</td>
<td>58.19 ± 0.66</td>
<td>55.64 ± 0.53</td>
<td>51.75 ± 0.37</td>
<td>49.31 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>59.00 ± 0.77</td>
<td>57.96 ± 0.85</td>
<td>54.79 ± 0.54</td>
<td>53.00 ± 0.47</td>
<td>49.13 ± 0.92</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>Air</td>
<td>20.71 ± 0.24</td>
<td>19.70 ± 0.18</td>
<td>19.01 ± 0.25</td>
<td>18.18 ± 0.11</td>
<td>17.14 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>20.47 ± 0.16</td>
<td>19.54 ± 0.29</td>
<td>18.71 ± 0.16</td>
<td>18.30 ± 0.12</td>
<td>16.68 ± 0.55</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>Air</td>
<td>34.76 ± 0.38</td>
<td>33.87 ± 0.21</td>
<td>34.20 ± 0.43</td>
<td>35.16 ± 0.38</td>
<td>34.75 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>34.70 ± 0.39</td>
<td>33.70 ± 0.29</td>
<td>34.24 ± 0.29</td>
<td>34.54 ± 0.39</td>
<td>33.94 ± 0.57</td>
</tr>
<tr>
<td>RDW-CV (%)</td>
<td>Air</td>
<td>14.71 ± 0.17</td>
<td>14.99 ± 0.31</td>
<td>16.14 ± 0.47</td>
<td>16.78 ± 0.74</td>
<td>19.49 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>14.42 ± 0.31</td>
<td>15.55 ± 0.56</td>
<td>15.93 ± 0.38</td>
<td>17.71 ± 0.60</td>
<td>20.10 ± 0.78</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>Air</td>
<td>323.79 ± 19</td>
<td>304.61 ± 14</td>
<td>222.24 ± 15</td>
<td>197.94 ± 14</td>
<td>201.98 ± 12</td>
</tr>
<tr>
<td>(K/μL)</td>
<td>ABS</td>
<td>311.38 ± 33</td>
<td>324.55 ± 13</td>
<td>215.20 ± 11</td>
<td>221.35 ± 10</td>
<td>243.14 ± 10</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>Air</td>
<td>6.06 ± 0.19</td>
<td>4.60 ± 0.19</td>
<td>3.08 ± 0.24</td>
<td>2.73 ± 0.05</td>
<td>2.86 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>5.74 ± 0.23</td>
<td>4.82 ± 0.25</td>
<td>3.21 ± 0.32</td>
<td>2.96 ± 0.04</td>
<td>3.05 ± 0.14</td>
</tr>
<tr>
<td>Platelets</td>
<td>Air</td>
<td>566.67 ± 56</td>
<td>765.80 ± 53</td>
<td>643.43 ± 158</td>
<td>438.20 ± 167</td>
<td>415.50 ± 109</td>
</tr>
<tr>
<td>(K/μL)</td>
<td>ABS</td>
<td>415.67 ± 102</td>
<td>700.20 ± 98</td>
<td>592.17 ± 137</td>
<td>838.67 ± 83*</td>
<td>675.80 ± 89</td>
</tr>
<tr>
<td>PDW (fL)</td>
<td>Air</td>
<td>8.30 ± 0.27</td>
<td>8.28 ± 0.06</td>
<td>8.36 ± 0.25</td>
<td>8.44 ± 0.45</td>
<td>8.47 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>8.00 ± 0.60</td>
<td>8.43 ± 0.22</td>
<td>8.78 ± 0.34</td>
<td>7.95 ± 0.16</td>
<td>8.39 ± 0.14</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>Air</td>
<td>7.00 ± 0.17</td>
<td>7.30 ± 0.05</td>
<td>7.16 ± 0.17</td>
<td>7.22 ± 0.25</td>
<td>7.07 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>6.75 ± 0.65</td>
<td>7.28 ± 0.08</td>
<td>7.42 ± 0.20</td>
<td>6.83 ± 0.11</td>
<td>7.05 ± 0.16</td>
</tr>
</tbody>
</table>
Table 3.5. Hematological parameters. Values represents means ± SEMs; n = 8/group/time point. *p<0.05 versus air-control group.

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.26 ± 0.72</td>
<td>5.01 ± 0.64</td>
<td>4.41 ± 0.66</td>
<td>4.31 ± 0.57</td>
<td>3.75 ± 0.64</td>
</tr>
<tr>
<td><strong>Leukocytes (K/μL)</strong></td>
<td>2.44 ± 0.65</td>
<td>5.71 ± 0.76</td>
<td>4.59 ± 0.48</td>
<td>4.46 ± 0.47</td>
<td>3.93 ± 0.64</td>
</tr>
<tr>
<td><strong>% Neutrophils</strong></td>
<td>6.08 ± 1.12</td>
<td>8.79 ± 1.72</td>
<td>9.82 ± 0.90</td>
<td>8.80 ± 0.73</td>
<td>13.11 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>4.77 ± 0.87</td>
<td>8.59 ± 0.82</td>
<td>9.78 ± 1.72</td>
<td>11.85 ± 1.18</td>
<td>15.84 ± 1.86</td>
</tr>
<tr>
<td><strong>% Lymphocytes</strong></td>
<td>89.34 ± 1.59</td>
<td>87.17 ± 2.47</td>
<td>84.86 ± 1.56</td>
<td>87.13 ± 0.88</td>
<td>80.24 ± 2.98</td>
</tr>
<tr>
<td></td>
<td>92.58 ± 1.32</td>
<td>86.45 ± 1.32</td>
<td>85.15 ± 2.51</td>
<td>80.56 ± 2.51</td>
<td>77.38 ± 2.23</td>
</tr>
<tr>
<td><strong>% Monocytes</strong></td>
<td>2.42 ± 0.22</td>
<td>2.37 ± 0.65</td>
<td>2.50 ± 0.57</td>
<td>2.20 ± 0.28</td>
<td>1.56 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>1.65 ± 0.19</td>
<td>2.21 ± 0.25</td>
<td>1.85 ± 0.40</td>
<td><strong>3.80 ± 0.51</strong></td>
<td>2.18 ± 0.38</td>
</tr>
<tr>
<td><strong>% Eosinophils</strong></td>
<td>1.24 ± 0.27</td>
<td>1.56 ± 0.54</td>
<td>3.05 ± 0.90</td>
<td>1.71 ± 0.19</td>
<td>5.66 ± 1.92</td>
</tr>
<tr>
<td></td>
<td>1.16 ± 0.37</td>
<td>1.60 ± 0.28</td>
<td>3.04 ± 0.97</td>
<td>2.60 ± 0.76</td>
<td>4.35 ± 0.87</td>
</tr>
<tr>
<td><strong>% Basophils</strong></td>
<td>0.30 ± 0.03</td>
<td>0.27 ± 0.07</td>
<td>0.28 ± 0.06</td>
<td>0.16 ± 0.07</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.18 ± 0.02</td>
<td>0.27 ± 0.04</td>
<td>0.25 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>Marker</td>
<td>Treatment</td>
<td>Exposure duration (days)</td>
<td>Standard ranges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>--------------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>CREA (mg/dL)</td>
<td>Air</td>
<td>0.13 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.20 ± 0.03</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>0.10 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>Air</td>
<td>15.57 ± 0.65</td>
<td>15.88 ± 0.81</td>
<td>16.63 ± 0.50</td>
<td>14.75 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>15.50 ± 1.29</td>
<td>13.88 ± 0.93</td>
<td>18.63 ± 1.09</td>
<td>17.88 ± 0.93*</td>
</tr>
<tr>
<td>PHOS (mg/dL)</td>
<td>Air</td>
<td>9.89 ± 0.31</td>
<td>8.68 ± 0.13</td>
<td>8.41 ± 0.41</td>
<td>7.98 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>10.93 ± 0.49*</td>
<td>9.26 ± 0.23</td>
<td>9.05 ± 0.26</td>
<td>9.51 ± 0.51*</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>Air</td>
<td>5.24 ± 0.16</td>
<td>5.25 ± 0.05</td>
<td>5.23 ± 0.10</td>
<td>5.38 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>5.37 ± 0.10</td>
<td>5.28 ± 0.07</td>
<td>5.24 ± 0.12</td>
<td>5.48 ± 0.14</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>Air</td>
<td>2.80 ± 0.15</td>
<td>2.71 ± 0.04</td>
<td>2.73 ± 0.08</td>
<td>2.74 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>2.85 ± 0.07</td>
<td>2.74 ± 0.05</td>
<td>2.68 ± 0.08</td>
<td>2.86 ± 0.09</td>
</tr>
<tr>
<td>GLOB (g/dL)</td>
<td>Air</td>
<td>2.44 ± 0.02</td>
<td>2.54 ± 0.04</td>
<td>2.50 ± 0.05</td>
<td>2.64 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>2.52 ± 0.03</td>
<td>2.54 ± 0.05</td>
<td>2.56 ± 0.50</td>
<td>2.61 ± 0.07</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>Air</td>
<td>63.57 ± 11.12</td>
<td>55.50 ± 5.05</td>
<td>59.38 ± 6.07</td>
<td>51.63 ± 3.35</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>64.33 ± 5.41</td>
<td>50.25 ± 3.50</td>
<td>61.88 ± 10.95</td>
<td>47.70 ± 7.23</td>
</tr>
<tr>
<td>ALKP (U/L)</td>
<td>Air</td>
<td>215.86 ± 15</td>
<td>227.25 ± 9</td>
<td>186.88 ± 12</td>
<td>184.13 ± 11</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>215.17 ± 18</td>
<td>244.38 ± 18</td>
<td>212.38 ± 17</td>
<td>215.17 ± 18</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>Air</td>
<td>0.31 ± 0.14</td>
<td>0.14 ± 0.03</td>
<td>0.13 ± 0.03</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>0.52 ± 0.11</td>
<td>0.15 ± 0.04</td>
<td>0.15 ± 0.05</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>CHOL (mg/dL)</td>
<td>Air</td>
<td>50.29 ± 3.00</td>
<td>47.250 ± 1.26</td>
<td>39.38 ± 3.54</td>
<td>43.00 ± 3.99</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>50.00 ± 3.00</td>
<td>49.57 ± 1.66</td>
<td>36.25 ± 4.86</td>
<td>46.88 ± 2.17</td>
</tr>
<tr>
<td>CK (U/L)</td>
<td>Air</td>
<td>200.80 ± 65</td>
<td>92.00 ± 7</td>
<td>102.50 ± 21</td>
<td>105.70 ± 12</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>457.37 ± 64*</td>
<td>120.80 ± 16</td>
<td>118.53 ± 14</td>
<td>108.53 ± 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>LDH</strong> (U/L)</td>
<td><strong>URIC</strong> (mg/dL)</td>
<td><strong>AST</strong> (U/L)</td>
<td><strong>NH3</strong> (µmol/L)</td>
<td><strong>CRP</strong> (mg/dL)</td>
<td></td>
</tr>
<tr>
<td><strong>Air</strong></td>
<td>326.55 ± 45</td>
<td>1.64 ± 0.67</td>
<td>57.71 ± 16.95</td>
<td>0.17 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>ABS</strong></td>
<td>649.80 ± 97*</td>
<td>0.68 ± 0.07</td>
<td>31.00 ± 7.04</td>
<td>0.16 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>Air</strong></td>
<td>388.04 ± 56</td>
<td>1.33 ± 0.87</td>
<td>27.75 ± 4.02</td>
<td>0.19 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>ABS</strong></td>
<td>383.01 ± 65</td>
<td>0.50 ± 0.08</td>
<td>25.88 ± 3.76</td>
<td>0.20 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>Air</strong></td>
<td>322.51 ± 90</td>
<td>0.45 ± 0.05</td>
<td>30.43 ± 5.71</td>
<td>0.19 ± 0.03</td>
<td></td>
</tr>
<tr>
<td><strong>ABS</strong></td>
<td>335.49 ± 42</td>
<td>1.60 ± 0.59*</td>
<td>30.43 ± 5.71</td>
<td>0.16 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.6. Serum chemistry profiles.** Values represents means ± SEMs; n = 8/group/time point. *p<0.05 versus air-control group.
3.3.5. Histopathology of lung and nasal passages

H&E-stained sections of the left lung lobe and four levels of nasal passages (T1, T2, T3, and T4) were examined for routine histopathology using light microscopy (Olympus BX53). Tissue sections from air-exposed control animals were also examined for determining background pathology. Exposure-related changes were not observed in any lung or nasal passage sections examined for any treatment group.

3.4. DISCUSSION

From industrial, educational to medical and healthcare applications, 3-D printing proves to be an exciting and promising technology [103]. However, as with any emerging technology, little is known about the toxicity of the emissions generated during printing and how emissions may differ from various types of raw material.

In our study, the ABS-emissions generated during real-time printing with a consumer-grade level 3-D printer included incidental UFPs and VOCs. The temporal pattern and size distributions (including peak, average number, and mass concentrations) of particle emissions were characterized during animal exposures to compare to real-world exposure settings. Particle number concentration in the exposure chamber followed a temporal pattern that consisted of a rapid rise during the first few min of printing, followed by a slower decline throughout the remainder of the print time. A similar pattern was reported for indoor environments and industrial workplaces where FFF 3-D sprinters extrude ABS filament. During animal exposures, peak particle concentrations were about $10^6$ particles/cm$^3$, which is a factor of 15 to 25 higher than levels observed in industrial workplaces where FFF 3-D printers extrude ABS filament. The daily average particle number concentration during animal exposures was $88,000 \pm 23,000$ particles/cm$^3$. This daily average value is similar to that reported for a poorly ventilated college dormitory [275] and a Finnish workplace [113], but a factor of three to five higher than reported for other industrial workplaces [322]. Geometric mean particle diameter was about 40 nm at the start of printing and increased to 80 nm as printing progressed. Although data on aerosol size distribution in occupational environments are sparse, our observed size is
similar to that reported for a non-industrial indoor environment. Given their small size, UFPs from FFF 3-D printers have a low mass. Vaisanen et al. [113] measured, via a real-time monitor, a particle mass concentration of 10 µg/m³ in a workplace. McDonnell et al. [275], also using a real-time monitor, calculated particle mass concentrations ranging from 120 to 620 µg/m³ for a dormitory room and small office on a college campus.

Our previous in vitro study delivered an average particle concentration of $1 \times 10^7$ ABS particles per ml, which would result in an equivalent alveolar lung burden of ABS particles of 758 µg in the rat. In our current study, the day 30 alveolar deposition estimates were 25.54 µg and 12.10 µg, representing the model without and with clearance, respectively, which is about 30 to 60 times lower than the in vitro study. As such, we attribute the minimal respiratory and systemic changes observed in this study to lower particle deposition than that delivered in the in vitro study.

In terms of VOCs concentrations, we found that all detected VOCs emitted over 4-h print jobs were at average levels that were much lower than workplace exposure limits (REL or PEL), whose values are appropriate for comparison to exposure measurements collected in occupational settings and would not be protective of sensitive individuals or children in non-occupational settings. We identified styrene, benzene, and acetaldehyde vapor, which may irritate the mucous membranes of the respiratory tract [323, 324]. An important finding from previous studies is that filament factors such as the formulation, the temperature to which it is heated, and the brand influences its breakdown and resultant released gas profile. Wojtyla et al. [102] identified more than 70 different VOCs in ABS filament emissions, with styrene, ethylbenzene, benzaldehyde, formaldehyde, acetophenone, and vinyl cyclohexene as the most abundant. Another study [107] found that printing with ABS filaments released ethylbenzene at levels 16.4 times higher than outdoor air concentrations, isovaleraldehyde 11.9 times higher, and acetaldehyde 3.2 times higher. More recently, Davis et al. [122] detected 177 individual VOCs during FFF 3-D printing with ABS. These studies substantiate the potential for human exposure and subsequent health effects from VOCs emitted during printing. Technologies to reduce and prevent VOC emissions from FFF 3-D printing are emerging. When testing the efficiency of commercially available filter covers and air-purifiers, varying capabilities of these
control measures in removing VOCs were observed. It was found that conditioning the new filters and pre-operating the control devices for a few days helped reduce VOCs emissions. However, these control measures were unable to eliminate VOCs completely and even produced new types of VOCs, demonstrating that the development of effective control strategies for reducing VOC emissions could be more problematic. Recently, an advanced filtration system such as photocatalytic filtration has been created. It was based on doped graphitic carbon nitride materials and was able to successfully decompose and therefore reduce the emission of hazardous VOCs [325].

As noted in our study and previously reported by others [93, 113], most incidental particles emitted from FFF 3-D printing are smaller than 100 nm. Therefore, they have the potential to penetrate deeper into the lungs than larger particles and trigger local inflammatory and oxidative stress effects by activating resident macrophages and epithelial cells [326]. To evaluate the pulmonary effects of FFF ABS-emissions, we measured inflammatory and injury markers in BALF (cell differential, total protein, LDH activity, surfactant proteins A and D, and pro- and anti-inflammatory cytokines and chemokines) and in lung homogenates (the lipid peroxidation-derived malondialdehyde and protein carbonyl). Furthermore, sections of the left lung lobe and four levels of nasal passages were examined for histopathological changes. We found that after one day of exposure to ABS-emissions, the levels of IFN-γ, one of the most potent macrophage activators, spiked significantly in BALF and decreased thereafter. At day one of exposure, we also noted a slight increase in TNF-α, a cell-signaling cytokine produced by activated macrophages and involved in acute systemic inflammation, and IL-6, a cytokine with both pro-inflammatory and anti-inflammatory functions. However, the number of alveolar macrophages in BALF didn't increase significantly until the 15th day of exposure, when we also found that a decrease in IFN-γ levels correlated with increased secretion of the anti-inflammatory cytokine IL-10. We speculate that at earlier time points, the macrophages were activated by the pro-inflammatory mediators IFN-γ and TNF-α released in response to subtle injuries, resulting in the macrophages adhere to the alveolar lung wall, as previously reported [327, 328]. To counteract the tissue-damaging potential of the pro-inflammatory state of "classically activated" [329] macrophages (M1 phenotype), a switch to an anti-inflammatory or reparative state, "alternatively activated"
or M2 phenotype [330], occurred stimulated by the production of anti-inflammatory markers, such as IL-10.

This injury-repair response may explain the "delayed" influx of alveolar macrophages at the damaged site and its peak occurrence in BALF at the 15th day of exposure, which corresponded with increased blood monocytes and platelets. These results tie to previous studies [331, 332] that demonstrate that inflammatory signals (e.g., cytokines) produced by alveolar macrophages after phagocytosis of particles can stimulate the release of monocytes from the bone marrow into the circulation. Monocytes that are attracted to the injured site by growth factors released by platelets enter the lung through blood vessel walls and mature into macrophages [333]. Increased platelet count is a notable finding as it is also associated with cardiovascular effects [334-336], suggesting a possible link between FFF 3-D printer emissions and cardiovascular events. A previously published study observed that exposure to 3-D printer emissions stimulates acute hypertension and microvascular dysfunction.

Surfactant proteins SP-A and SP-D play a critical role in lung host defense by inhibiting inflammation, enhancing the elimination of pathogens and foreign particles, and influencing overall surfactant homeostasis and disease progression [337]. Recent advances in lung pathogenesis and understanding the roles of these two pulmonary collectins suggest that SP-A and SP-D may potentially serve as biomarkers of disease or injury [338, 339]. In our study, we found slightly decreased SP-A (day 8) and SP-D (day 4) levels in BALF. Decreased concentrations of surfactant proteins in BALF were reported as a result of long-term smoking [340, 341] or as an age-related effect [341-343], perhaps indicating pulmonary damage due to loss of alveolar epithelium integrity.

Lung endothelial and epithelial cells form a functional air-blood barrier that is important for rapid and effective gas exchange between alveoli and microvasculature, and for preventing translocation of micron-size inhaled particles into the circulatory system. However, the nanometer size of particles emitted during additive manufacturing may facilitate penetration of the air-blood barrier, and result in adverse health effects, as shown for polystyrene nanoparticles. To further explore the potential systemic toxicity of
ABS-emitted particles, we evaluated serum chemistry biomarkers involved in inflammatory and injury processes, as well as biomarkers of muscle, metabolic, liver, and kidney function. Among the 16 markers analyzed, the only significant changes above normal ranges in ABS-treated rats occurred on day 1 of exposure with an increase in creatinine kinase and aspartate aminotransferase, markers of kidney and hepatic function, respectively. A significant increase in other markers of hepatic (day 1) and renal (day 15) damage was noted. However, these changes fell within normal ranges, and returned to air-control levels by day 30 of exposure, indicating a transient systemic toxic effect. Pulmonary findings are consistent with the blood analysis, suggesting that the changes caused by exposure to ABS-emissions, at the durations and dose studied, are minimal and transitory. A similar pattern was observed in a previous study, which evaluated the effects of thermo-oxidative degradation products of heated poly(acrylonitrile-butadiene-styrene) at 300 – 330 °C. Male Wistar rats were exposed to a single concentration of degradation products for 1, 3, and 10 days (6 h/night, 5 nights/week), and 3 h post-exposure animals were assessed for adverse effects on lung, liver, kidney, and brain. The degradation products included styrene (11 – 24 ppm), carbon monoxide (4-5 ppm), formaldehyde (0.20 - 0.31 ppm), hydrogen cyanide (0.08 – 0.67 ppm), acrolein (1.4 -1.9 ppm), acrylonitrile (2 - 4 ppm), and aerosol fraction (8 - 26 mg/m³). The authors observed that exposure caused a significant reduction of 7-ethoxycoumarin O-deethylation activity (a marker for assessing substrate specificities of cytochrome P450) in the lung and kidney, but not in the liver, after one day of exposure. At the same exposure endpoint, a decrease in reduced glutathione concentration (GSH) in the liver and kidney, but not in the lung, was noted, suggesting decreased antioxidant defense. After 3 days of exposure, lung GSH levels remained decreased and returned to control levels by day 10.

At this time, it is unclear if particle number, mass, or surface area is the most relevant metric of toxicity for FFF 3-D printer emissions. Gumperlein et al. [273] exposed human volunteers to ABS emissions at 1.6 × 10⁶ particles/cm³ and reported minimal evidence of inflammatory responses based on biomonitoring. Specifically, the volunteers' exhaled air showed increased levels of nitric oxide, which might be due to inhaled ultrafine particle-induced eosinophilic inflammation. However, no clinically significant acute
changes in the biochemical responses of nasal secretions and urine were found. In an in vivo toxicology study, Stefaniak et al. [223] exposed rats once to ABS emissions at 0.9 ± 0.1 mg/m³ (Dₚ ~ 70 nm) and observed acute hypertension. Hence, caution is needed when interpreting the results of the current inhalation study because the lack of significant responses for several toxicological outcomes may not prove the absence of ABS toxicity, but rather be an effect from 1) the choice of exposure metric and/or 2) the relatively low particle number and mass exposures compared to those employed in other toxicology studies. While the temporal pattern of particulate emissions in the exposure chamber was in the range or higher than observed in real-world settings [113, 275], a significant challenge for this and future inhalation toxicology studies of FFF 3-D printer emissions is the generation of consistent daily aerosol exposure with number and mass concentration characteristics that are higher than observed in the real-world workplace to establish a minimal effect and no effect level. Furthermore, careful selection of the most appropriate and relevant animal species is essential considering the differences in the anatomy of the airways, the characteristics of particle deposition and clearance, which would ultimately have implications in the observed health effects. In addition, a dose-dependence relationship would be relevant to establish some criteria to base the relative safety of FFF 3-D printer emissions.

3.5. CONCLUSIONS

Repeated whole-body inhalation exposures to FFF 3-D printing emissions (particles at 240 ± 90 µg/m³ concentration, and the listed VOC types at the concentrations reported) released from ABS filament in commercially available desktop 3-D printers for 4 h/day for 1, 4, 8, 15, and 30 days of exposure (4 days/week) caused minimal and transient pulmonary and systemic effects to rats. Our findings suggest that ABS emissions elicited an adaptive response in this exposure paradigm. This adaptive phenomenon refers to when a low stimulating dose of stress is administered that initiates compensatory biological processes [344]. However, considering the significant variability observed in the emission characteristics for one ABS filament and one printer model combination, to multiple printers, filament and operation condition combinations, we
cannot exclude the potential of further acute and chronic inflammatory responses, particularly when human case studies report the development of respiratory symptoms and new diagnoses of asthma and allergic rhinitis from workplace 3-D printer exposures [267, 268].

Funding

This investigation was supported by U.S. Consumer Product Safety Commission (CPSC) and the National Institute for Occupational Safety and Health (NIOSH), Project [093909NF].

Disclosure of interest

The authors report no conflict of interest.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of brand name does not constitute product endorsement.

This work has not been reviewed or approved by and does not necessarily represent the views of, the Commission.
Supplemental Figure 3.1. Object printed for each exposure and sample run: A) completed 3-D printed object, and B) 3-D printed object stopped before printing the top layers to show infill pattern. The objects printed were 12.7 cm wide by 12.7 cm long, with a height of about 2.54 cm.
Supplemental Figure 3.2. Experimental design. The 4 hours per day exposure duration was selected due to time issues. This was the maximum amount of time that could be allocated to our study by the NIOSH Inhalation Facility staff without impacting other inhalation exposure projects conducted at the same time. Prior to each exposure, the staff had to clean the chambers, remove old, printed parts, clean build plates, replace clogged print heads, load / unload animals into the two different exposure chambers (HEPA-filtered air and printer emissions-exposure chamber, respectively), load the filaments into the three printers, ensure proper printing, etc. The 4 days per week exposure schedule was selected to accommodate the schedule of our collaborators who were involved in FFF 3-D printer emission-induced neurotoxicity, hepatotoxicity, microvascular, cardiovascular, and reproductive toxicities studies.
Two identical exposure chambers were used to simultaneously conduct the exposures to HEPA-filtered air and ABS emissions, respectively. The exposures were performed during the daylight portion of the rats’ circadian cycle (from about 9:00 am until 1:00 pm), when they would be mostly inactive/sleeping. Also, during this time, the animals did not have access to food/water or bedding.

Uniformity of exposure from one animal to another was achieved by rotating the rat’s locations daily, throughout the exposure sessions within the animal cage. Furthermore, based on previous tests conducted on the same exposure chambers, we observed less than a 12% concentration variation across animal cage locations, indicating a spatial uniformity of airborne distribution of printer emissions.

The aerosol mass concentration inside the exposure chamber was continuously monitored with a Data RAM (DR-40000; Thermo Electron Co., Waltham, MA), which was connected to a 1/4 inches (0.64 cm) stainless steel sample tube to sample air from a location near the animals' breathing space. The 240 µg/m³ exposure concentration was the maximum concentration that we could reliably achieve for the repeated exposure runs. Based on our previous experience and other researcher’s reports [93], the inherent variability in the ultrafine particulate (UFP) between the repeated print runs using the same filament is recognized and unavoidable at the moment.

We have not evaluated the particle size or transformations/agglomerations pre-tube (inside printer generation chamber) versus post-tube (inside animal exposure chamber). However, the tubing length was about 6 inches (15 cm) long. Therefore, the particle agglomeration could be affected more by the time spent in the 3-D printer enclosure or time in the exposure chamber. Moreover, the particle size range generated with our system is similar to what has been reported in the literature, suggesting that our system does not affect the particle size and is relevant to indoor environments and occupational settings.
Supplemental Figure 3.3. Body weight of animals at euthanasia demonstrating that the rats were gaining weight over time, and that there is no significant difference between the air-control and ABS-treated group.

*Estimating equivalent alveolar lung burden in a rat based on previous cell exposures*

For comparison of results in the current study, calculations were made to estimate the equivalent lung burden required for rat lungs to achieve the same dose as that given to the cell culture.

The following calculations were made using a custom MATLAB script (MathWorks Inc., Natick, MA). In the cell exposure study, the median particle size in the cell culture medium was found to be 201 nm and ranged in size from about 100 nm to 400 nm. A log-normal distribution curve was generated with a median of 200 nm and to have 95% of its total area between 100 and 400 nm. This distribution had a bin (data point) every 10 nm ranging from 50 to 600 nm. The total sum of all the bins was set to $1 \times 10^7$. This was meant to represent one ml of a solution containing $10^7$ particles. In our previous study, the ABS average particle concentration per ml ranged from $0.45 \times 10^7$ to $1.51 \times 10^7$. The $10^7$ value was used because it corresponded to one of the doses previously measured and tested in the cell culture media and for easier back-calculation. Assuming all particles are spherical and have a density of 1.04 g/cm$^3$ (density of ABS filament), the mass distribution can be calculated. Each data point in the count distribution was converted to mass using the following equation:
Mass = D x (4/3) x π x r³

Where: D = density, and r = radius of the particle. The sum of mass from every bin in the distribution was then calculated, resulting in 0.626 µg/ml of solution. Each well was given 0.100 ml of solution. Thus, the estimated particle mass per cell well was 0.0626 µg. Each cell well had a surface area of 0.33 cm². By dividing mass per cell well by area of well, the result was 0.190 µg/cm². A typical rat lung has an alveolar surface area of approximately 4,000 cm². To achieve the same mass per surface area in a rat lung as what was calculated to be on the wells, a rat would have a lung burden of (0.190 µg/cm²) x (4,000 cm²) = 759 µg.

Summary of MATLAB calculations
mass per ml per 1 x 10⁷ particles in solution = 0.626 µg
each cell well was given 0.100 ml of solution.
mass per well = 0.062606 µg
each cell well area = 0.33 cm²
mass per area on cells = 0.189716 µg/cm²
area of a typical rat lung = 4,000 cm²
equivalent lung burden in a rat for 10⁷ particles = 758.8 µg

Based on MATLAB calculations, the equivalent alveolar lung burden in a rat for 10⁷ ABS particles per ml would be equal to approximately 758 µg in this study.
Supplemental Figure 3.4. Figure adapted from our previous study displaying the regression analyses performed on the cell viability and LDH activity of small airway epithelial cells using particle number*10^{-4} as the independent variable. The dotted black line indicates 3.5 \times 10^6 particle/cm^2, which is approximately equal to 1 \times 10^7 ABS particles per ml, which caused a 10% decrease in viability and 20% increase of LDH over control.
Supplemental Figure 3.5. IgE serum levels. The rats were exposed for 1, 4, 8, 15, and 30 days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represents means ± SEMs; N = 8/group/time point.
<table>
<thead>
<tr>
<th>Setting Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Print nozzle temperature</td>
<td>260 °C</td>
</tr>
<tr>
<td>Print bed temperature</td>
<td>100 °C</td>
</tr>
<tr>
<td>Layer Height</td>
<td>0.25 mm</td>
</tr>
<tr>
<td>Line Width</td>
<td>0.5 mm</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>3 lines (1.5 mm)</td>
</tr>
<tr>
<td>Number of solid bottom layers</td>
<td>4 (1.0 mm)</td>
</tr>
<tr>
<td>Number of solid top layers</td>
<td>4 (1.0 mm)</td>
</tr>
<tr>
<td>Infill density</td>
<td>25%</td>
</tr>
<tr>
<td>Print speed (1st layer)</td>
<td>45 mm/sec</td>
</tr>
<tr>
<td>Print speed after 1st layer</td>
<td>120 mm/sec</td>
</tr>
<tr>
<td>Print bed adhesion</td>
<td>PEI sheet, no glue.</td>
</tr>
</tbody>
</table>

**Supplemental Table 3.1. Three-dimensional printer settings.**

Acrylonitrile butadiene styrene (ABS) is one of the most popular filaments used in FFF 3-D printers. It is a petrochemical material derived from the copolymerization of 1,3-butadiene and styrene. The butadiene units offer good impact strength, the styrene units give the copolymer its rigidity, and the acrylonitrile units allow heat resistance, making it widely used in industry [345].
Supplemental Table 3.2. Serum cytokines in rats following exposure to ABS emissions at any time point when compared to air-control rats. The rats were exposed for 1, 4, 8, 15, and 30 days to air or ABS emissions (240 µg/m³) and euthanized at 24 h post last exposure. Values represents means ± SEMs; N = 8/group/time point.

<table>
<thead>
<tr>
<th>Marker (pg/ml ± SEM)</th>
<th>Agent</th>
<th>Exposure duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Air</td>
<td>14.87 ± 1.50</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>13.92 ± 1.28</td>
</tr>
<tr>
<td>IL-10</td>
<td>Air</td>
<td>61.55 ± 3.77</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>61.53 ± 3.273</td>
</tr>
<tr>
<td>IL-13</td>
<td>Air</td>
<td>4.28 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>3.66 ± 0.47</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Air</td>
<td>5.10 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>3.87 ± 0.01</td>
</tr>
<tr>
<td>IL-4</td>
<td>Air</td>
<td>8.48 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>8.98 ± 0.67</td>
</tr>
<tr>
<td>IL-5</td>
<td>Air</td>
<td>46.43 ± 7.13</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>44.58 ± 4.30</td>
</tr>
<tr>
<td>IL-6</td>
<td>Air</td>
<td>79.59 ± 9.69</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>70.72 ± 11.83</td>
</tr>
<tr>
<td>KC/GRO</td>
<td>Air</td>
<td>267.57 ± 28.59</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>258.51 ± 16.41</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Air</td>
<td>5.74 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>ABS</td>
<td>5.81 ± 0.55</td>
</tr>
</tbody>
</table>
CHAPTER 4: EVALUATION OF PULMONARY EFFECTS OF ACRYLONITRILE BUTADIENE STYRENE (ABS) 3-D PRINTER EMISSIONS USING AN AIR-LIQUID INTERFACE (ALI) MODEL OF HUMAN PRIMARY BRONCHIAL EPITHELIAL (NHBE) CELLS

ABSTRACT
To date, the potential toxicity of aerosolized emissions from FFF 3-D printers has been tested in vitro in conventional submerged single-cell culture and in rodents. Considering the dosimetry drawbacks and lack of the physiological features of airway mucosa when using submerged in vitro models as well as challenging data extrapolation from animals to humans due to respiratory tract interspecies variability, efforts focused on developing human-relevant models, with particular emphasis on in vitro models. This study aimed to investigate the use of primary normal, human-derived bronchial epithelial cells (NHBEs) as in vitro inhalation toxicity model of lung epithelium to mimic and study the behavior of respiratory epithelium upon exposure to emissions from FFF 3-D printing with acrylonitrile butadiene styrene (ABS) filament. NHBEs epithelium integrity and differentiation changes, cytotoxicity, tissue injury, and inflammatory and immune system regulation markers were evaluated following 4 hrs. exposure to ABS filament emissions. The mean diameter and the mode diameter of ABS particle in the medium was 170 ± 21 nm, and 144 ± 15 nm, respectively. The average ABS particle deposition per surface area of the epithelium was 1.7 x 10^7 ± 1.6 x 10^7 particle/cm². Overall, at the conditions applied, exposure of NHBEs to ABS emissions did not affect epithelium integrity, ciliation, mucus production, or induce cytotoxicity. At 24 hrs. after the exposure, significant increases in IL-12p70, IFN-γ, TNF-α, IL-17A, VEGF, and MIP-1α were noted in the basal cell culture medium of ABS-exposed cells compared to non-exposed chamber control cells. To identify potential threshold values and mechanistic studies, exposure to higher concentrations of AB emissions should be performed.

Keywords: 3-D printers, printer emitted nanoparticles, filament, air-liquid interface, in vitro, normal human bronchial epithelial cells.
4.1. INTRODUCTION

We are witnessing an accelerating expansion and evolution of additive manufacturing (AM). AM has a tremendous social, economic, and environmental impact providing numerous advantages in many areas [3, 6, 8, 23, 45, 50, 51, 60, 64, 77, 346, 347]. This revolutionary process is predicted to become a mainstream technology for serial production and automation [8]. However, considerable evidence [73, 85-96, 98-103] shows that the FFF 3-D printing process releases a significant number of nanoparticles (NPs), that can deposit deep in the lower respiratory tract and may cause adverse respiratory effects. The release of various volatile organic comounds (VOCs) might also pose serious risk concerns to human health as they are known irritants, carcinogens, odorants, and reprotoxins [110]. VOCs exposure can occur to gas phase substances as well as particle adhered VOCs.

The potential toxicity of aerosolized emissions from 3-D printers has been tested in conventional submerged single-cell culture [269, 348] and animal models [223, 269, 349]. However, the relevance of using submerged single cell in vitro models for performing cytotoxicity assessment of the respiratory system manifest drawbacks. First, these models do not mimic in vivo conditions such as physiological features of airway mucosa characterized by the presence of ciliated columnar cells, mucous-producing goblet cells, and basal cells [350]. Second, the addition of test agents in liquid form directly into the cell culture medium may alter its physicochemical properties, such as size, surface charge, solubility, transformation, or agglomeration state, leading to unreliable outcome or dose-response [351]. Third, there is an inability to recapitulate physiological inhalation conditions and aerosol deposition patterns comparative to in vivo human conditions [351]. Animal models, such as mice and rats, have been routinely used in experimental toxicology and risk/hazard assessments. However, data extrapolation from animals to humans is challenging because of respiratory tract interspecies variability. For example, such differences are based on 1) respiratory tract architectural features (e.g., nasal gross anatomy, airway structure, and branching pattern, the number of cartilaginous airways, cell types, and composition within the regions of the respiratory tract), 2) respiratory physiology (e.g., breathing mode and ventilatory patterns, metabolic rates),
and 3) biochemistry (e.g., composition and biotransformation capacity of P450 monooxygenases, epoxide hydrolase and glutathione S-transferase) [352-356]. Moreover, studies of translational toxicology from rats to humans in setting occupational exposure limits for hazard classification found that the extrapolation and standardization to rat lung mass or to rat body weight is varying and inconsistent [357], and the genomic responses in mouse models poorly mimic human inflammatory diseases [358]. Therefore, these variations influence the local toxic effects due to differences in the airflow pattern in the respective respiratory tract architecture, which affects the deposition of the given inhaled test agent as its retention and clearance from the lungs [359]. These limitations explain research efforts focused on developing human-relevant models, with particular emphasis on in vitro models [351, 360, 361].

Physiologically relevant respiratory epithelium models, such as in vivo-like in vitro models cultured at the air-liquid interface (ALI), have been established and routinely used to study inhaled particles or gases mediated respiratory toxicity [362]. These methods are promising in the domain of inhalation toxicology as they mimic the in vivo respiratory epithelium in its organization and stratification, avoiding limitations posed by the submerged monolayer cultures. This three-dimensional (3D) in vitro tissue model consists of normal, human-derived bronchial epithelial cells (NHBEs) cultured at the ALI on porous membrane inserts, and results in a polarized mucociliary differentiated airway epithelial cell layer. This in vitro epithelium is predominantly composed of three cell types, including mucus-producing goblet cells (secretory cells), ciliated respiratory epithelial cells, and basal cells (progenitor cells). This architecture mimics the biology of the in vivo respiratory tract by modeling epithelial barrier function (e.g., development of high trans-epithelial electrical resistance, expression and functionality of tight junctions, paracellular permeability), mucous production, and cilia function. Furthermore, the suitability of ALI cultures as benchmark for a human-relevant model has been further confirmed through transcriptome analyses [363], with several experiments demonstrating physiological responses to toxicants or pathogens [364-368]. Furthermore, due to intense ethical, societal, and legal support for the implementation of the "3R principle" (reduce, refine, and replace the number of animal use), the ALI approaches have been used successfully
as alternative methods for the replacement of animal-based inhalation toxicity studies [369].

Given that there are numerous types of filament on the market, and that the FFF 3-D printing emissions physicochemical characteristics vary with each filament type and within each type varies with color [119, 122], this might suggest that their toxic effects may vary as well in these conditions. Thus, ALI culture models provide a unique in vitro platform to assess the exposure to 3-D printer emissions avoiding the drawbacks of using submerged single cell in vitro models and animal models and would likely reduce the time and expenses of toxicity testing and screening of multiple filaments at once.

This study aimed to investigate the use of primary NHBEs as an in vitro inhalation toxicity model of lung epithelium to mimic the respiratory behavior upon exposure to FFF 3-D printer emissions. As such, we evaluated cytotoxicity, tissue injury, and inflammatory and immune system regulation markers following exposure to ABS filament, the most common filament on the market.

4.2. MATERIALS AND METHODS

4.2.1. Cell culture model

Primary NHBEs, isolated from the surface epithelium of human bronchi, were purchased from PromoCell (cat. C-12640, PromoCell GmbH, Germany). Cryopreserved cells (healthy donor number 446Z036.9 and 432Z016.5; viability ≥ 94%; 500,000 cells per vial) were cultured and differentiated according to the manufacturer’s instructions. Briefly, cells (passage P+1) were expanded in 1 x T75 Corning™ Cell Culture Treated Flasks, using the Pneumacult-Ex Plus expansion medium (cat. 05040, Stemcell Technologies, Vancouver, Canada), supplemented with 0.1% (v/v) hydrocortisone (cat. 07925, Stemcell Technologies, Vancouver, Canada). NHBE were cultured at 37 °C and under 5% CO₂ conditions, with a medium change every other day. After reaching 70% confluency, the cells were passaged into 4 x T75 Corning™ Cell Culture Treated Flasks (cell density at 0.5 x 10⁴ cells/cm²) using an Animal Component-Free Cell Dissociation Kit (Stemcell
Technologies, Vancouver, Canada) for 7-10 min at 37 °C. The detached cells (passage P+2) were centrifuged at 350 g for 5 min and re-suspended in the expansion medium. Cells were cultured for additional 4-5 days in the expansion medium, with medium change every other day, and subsequently used for the ALI procedure. To proceed with the ALI, 0.12 x 10^6 cells in 0.5 mL PneumaCult™-Ex Plus Medium were seeded on the apical side of inserts (0.4 μm pore membrane, Polyester, Costar® 12 mm Transwell®, Stemcell Technologies, Vancouver, Canada) in a 12-well plate with 1 mL expansion medium added into the basolateral side. The ALI condition was initiated 5 days post-seeding by the complete aspiration of the expansion medium in the apical compartment, thereby exposing the epithelial cells to the atmosphere (day 0 post-ALI) and replacing the medium from the basal compartment with the PneumaCult™-ALI Maintenance Medium (ALI medium). The ALI medium consisted of PneumaCult™-ALI Basal Medium containing 10% (v/v) PneumaCult™-ALI 10X Supplement, 1% (v/v) PneumaCult™-ALI Maintenance Supplement, 0.2% (v/v) heparin solution, 0.5% (v/v) hydrocortisone and 1% (v/v) Penicillin/Streptomycin. The cells were fed exclusively from the basal compartment with fresh ALI medium and maintained for 23 days before exposure to 3-D printer emissions.

4.2.2. Air-liquid interface cell exposure

4.2.2.1. Exposure system

The differentiated NHBEs cells were exposed to ABS 3-D printer emissions at the air-liquid interface using the exposure system utilized previously to expose Sprague Dawley rats to emissions generated during real-time printing via a whole-body inhalation exposure system. The 3-D printer emissions generation chamber and exposure chamber are described in detail in Chapter 3, 3.2.1 paragraph, pages 112-113.

4.2.2.2. Cell exposure treatment

Before cell exposure treatment, cell morphology was inspected using phase-contrast microscopy (Figure 4.1) followed by transepithelial electrical resistance (TEER) measurements, as described in the next paragraph.
Figure 4.1. Representative phase contrast microscopy image of NHBEs grown 23 days post-ALI.
Four different sets of treatments were employed, as follows: 1) cell inserts were placed in the middle of the chamber and exposed for 4 hrs. to the ABS FFF 3-D printer emissions (marked as ABS-exposed cells), 2) another set of cells on inserts were kept inside the emissions chamber covered, with the lid on (marked as “exposure chamber” samples), 3) the third set of cells were maintained in the incubator at normal growing conditions (marked as “incubator” samples), and 4) the last set of cells was treated on the apical surface of epithelial tissue with 0.1 ml 2% TX-100 (1 hr. prior to when the exposure ended) and used as a positive control for cytotoxicity and disruption of barrier integrity. After the 4 hrs. exposure treatment (time 0 hrs.) and 24 hrs. after exposure, various endpoints were evaluated, such as changes in phenotypic characteristics, functional changes, cytotoxicity, and inflammatory and immune system regulation markers (cytokines, chemokines, and growth factors). To check if the exposure might be a source of contamination, a plate containing cell culture medium without antibiotics was kept open in the exposure chamber during the 4 hrs. exposure. Next, the plate was maintained in the incubator for two weeks, inspected for contamination visually, and by trypan blue stain. After two weeks, the media was clear, no growth was observed, and no cells were present in the trypan blue-stained cell suspension.

4.2.2.3. Deposition characterization

During the 4 hrs. exposure to ABS-emissions, a 12-well Costar® Transwell® plate containing 0.5 ml PneumaCult™-Ex Plus Medium added on the apical side of 12 empty inserts (no cells) was kept inside the exposure chamber. After exposure, the cell culture medium was collected, stored in a sterile glass container, and used to characterize the ABS-emitted particles (mean hydrodynamic particle size and particle concentration) that would reach and deposit on the surface of the epithelial layer using nanoparticle tracking analysis (NTA) (NanoSight NS300, Malvern Instruments, Worcestershire, UK), as described in detail in Chapter 2, 2.2.2.1 paragraph, pages 71-72. The deposition of ABS-emitted particles was estimated by normalizing the number of ABS particles per mL measured in the collection medium using NTA to the surface area of the Transwell® inserts (1.12 cm²).
4.2.3. Evaluation of epithelial barrier integrity

As an important indicator of barrier integrity, TEER was measured using an Evom2 Epithelial Volt/Ohm meter equipped with 4 mm chopstick electrodes (World Precision Instruments Inc., Sarasota, FL). TEER was measured prior to treatment and at the end of the time point (0 and 24 hrs. after the exposure). To measure TEER, 0.5 mL of the corresponding fresh medium was added to the apical side of a 6-well insert, equilibrated for an additional 30 min at 37 °C, 5% CO₂, and then the chopstick electrodes were placed on either side of the primary airway epithelium. All TEER values were measured in duplicate for each well and corrected for cell-free insert (≈40 Ω) resistance and the surface area of a 12-well insert (1.12 cm²).

4.2.3. Video microscopy of cilia beating

The ciliary beating of live ALI tissue was visualized via phase-contrast microscopy before and after treatment using Revolve microscope (Echo Laboratories, San Diego, CA) at 40X magnification. The cilia movement was visualized prior to treatment and at the end of the time point.

4.2.4. Histology

Cultures were fixed in 4% (w/v) paraformaldehyde at room temperature until ready for processing. After fixation, the membrane inserts were rinsed twice with PBS. The samples were dehydrated using a series of increasing ethanol concentrations, cleared with xylene, infiltrated with paraffin wax, and embedded in wax blocks. Sections of 5 µm thickness were cut using a Thermo Scientific™ HM 325 Rotary Microtome (Thermo Fisher Scientific, Waltham, MA). The sections were stained with H&E or PAS using standard histological techniques and imaged using Olympus BX63 light microscope.

4.2.5. TEM analysis

For TEM analysis, the cells on the Transwell® membrane were fixed with 2% paraformaldehyde/ 2.5% glutaraldehyde solution (Karnovsky’s fixative) in sodium cacodylate buffer for at least 24 h, subsequently washed three times with 8% sucrose and 0.9% sodium chloride and post-fixed with 1% osmium tetroxide (2 hrs.), washed with
sucrose buffer, and stained *en bloc* with 1% tannic acid (1 hr). followed by 0.5% filtered uranyl acetate (1 hr). Afterwards, the cells were dehydrated in ascending ethanol series and embedded in epon. From the embedded cells, ultrathin sections were cut parallel to the vertical axis of the inserts, mounted on copper grids and stained with 4% uranyl acetate. The sections were imaged on a JEOL JEM 1010 Transmission Electron Microscope (JEOL, Tokyo, Japan) operating at 80kv with a side-mount digital AMT Orca camera system and software.

4.2.6. Immunofluorescence microscopy

Cultures were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature. The samples were incubated in 0.5 ml permeabilization buffer (0.5% [v/v] Triton X-100 in Dulbecco’s phosphate-buffered saline (D-PBS) for 15 min, washed once with 0.5 ml D-PBS and blocked for 1 h in 1% (w/v) bovine serum albumin (BSA) in D-PBS. Next, the cultures were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. The primary antibodies included rabbit anti-ZO-1 antibody (cat. 40-2200, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:100 dilution to identify tight junction formation, mouse anti-MUC5AC (45M1) antibody (cat. MA5-12178, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:200 dilution to identify the mucous cells, mouse anti-alpha tubulin antibody (B-5-1-2) (cat. 32-2500, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:200 dilution to identify cilia, and rabbit anti-e-cadherin (5HCLC) (cat. 710161, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:200 dilution to identify adherent junction. The samples were subsequently washed three times with 0.5 ml D-PBS and incubated with secondary antibodies and 1 µM Hoechst 33342 (cat. 62249, Thermo Fisher Scientific Inc., Waltham, MA) for 1 h at room temperature in the dark. The secondary antibodies included goat anti-mouse-Alexa Fluor 647 (cat. A-21236, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:500 dilution and goat anti-rabbit Alexa Fluor 488 (cat. A-11008, Thermo Fisher Scientific Inc., Waltham, MA) used at a 1:400 dilution. The samples were washed three times with 0.5 ml D-PBS, hold in 0.5 ml D-PBS until were visualized and imaged with Revolve microscope (Echo Laboratories, San Diego, CA).
4.2.7. Cytotoxicity

The possible cytotoxic effects of ABS emissions were assessed by measuring the cell viability and release of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium.

4.2.7.1. Cell viability assay

AlamarBlue™ Cell Viability Reagent (cat. DAL1025, Thermo Fisher Scientific, Waltham, MA), a non-toxic, cell-permeable compound, was used to assess cell viability. The assay was performed on the apical side as follows. After harvesting culture supernatants from the basolateral chamber for further analysis (LDH and cytokines analysis), 200 μL of 10% AlamarBlue solution was added to each insert, and fresh medium was supplemented in the basolateral chamber. After 4 h incubation at 37 °C, the viability was measured by quantifying fluorescence levels (ex/em 560/590 nm) using the Synergy H1 hybrid multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT).

4.2.7.2. LDH release assay

The supernatant from the basal chamber was collected, and the release of the cytosolic enzyme LDH into the medium, indicative of cell membrane damage, was assayed spectrophotometrically by monitoring the reduction of nicotinamide adenine dinucleotide at 340 nm in the presence of lactate using a Lactate Dehydrogenase Reagent Set (Pointe Scientific, Inc., Lincoln Park, MI).

4.2.8. Measurement of cytokine release

Media collected from the bottom (basolateral) wells were stored at – 80 °C until analysis. The concentrations of IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, MIP-3α, IL-17A/F, IL-17B, IL-17D, IL-3, IL-9, GM-CSF, IL-23p40, IL-15, IL-16, IL-17A, IL-1α, IL-5, IL-7, TNF-β, VEGF, IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α, Eotaxin, Eotaxin-3, IL-8(HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, and TARC (pg/ml) were measured using V-PLEX Human Biomarker Kits from Meso Scale Discovery (MSD, Meso Scale Discovery). Plates were read using MSD QuickPlex SQ 120 (Meso Scale Discovery).
Discovery) for electrochemiluminescence. Sample concentrations were derived from a standard curve plotted using a four-parameter logistic fit using MSD Workbench software.

4.2.9. Statistics

Studies were performed in one independent experiments with \( N = 1 - 3 \) inserts/treatment, \( n = 2 - 3 \) replicates each. The TEER, viability, and LDH data are represented as mean ± standard deviation. All statistical analyses were performed in JMP v13. Significant differences were determined by an ANOVA followed by the Tukey-Kramer multiple comparison procedure. All analyses are considered significant at \( p < 0.05 \).

The analysis of cytokines dataset began with checking the assumptions for two-way analysis of variance (ANOVA). The Normal Quantile Plot was used to assess the assumption of normality, while a Residual Plot was used to assess the equality of variances. The Levene’s test was also used to confirm the equality of variances. A logarithmic transformation of the response was used in the analysis if a residual plot exhibited a funnel shape and if the residual plot improved with the transformation. If the residuals of the response confirmed both assumptions, two-way ANOVA was performed. Any significant results in the two-way ANOVA table were further assessed with pooled t-tests to identify the specific main treatment effect and interaction effect. If a response did not pass the ANOVA assumptions, a Kruskal Wallis Test was performed on both main effects. Outliers in the data were identified using boxplots in R. To assess the significance of the outliers, a subset of the data was created without the identified outliers and analysis redone. If the outliers did not create a significant difference in the overall results, the outliers were included in the official analysis. If the outliers created a significant effect, the subset of the original data was used in the official analysis.

4.3. RESULTS

4.3.1. Particle characterization and deposition

Figure 4.2 presents the number-based size distribution of cell culture medium (background) and ABS 3-D printer-emitted particles (ABS1 - ABS4) collected in the cell culture medium analyzed using NTA.
**Figure 4.3.** Representative TEM image of ABS agglomerated printer-emitted particles on the surface of NHBEs cells 24 hrs. after the exposure.

The mean diameter and the mode diameter of ABS particle in the medium varied from 150 to 198 nm (170 ± 21 nm), and from 131 to 165 nm (144 ± 15 nm), respectively (Supplemental Table 4.1).

After background subtraction (plain cell culture medium, 3.6 × 10⁷ particles/insert), the average ABS particle concentration in medium ranged from 2.5 × 10⁵ to 4.4 × 10⁷ particles per insert (0.5 ml culture medium/1.12 cm²), which is equivalent to 2.2 × 10⁵ to 3.9 × 10⁷ (average 1.7 × 10⁷ ± 1.6 × 10⁷) ABS particles per unit surface area of the epithelium (Supplemental Table 4.2).

**4.3.2. Evaluation of NHBEs epithelial tight junction barrier integrity**

Before proceeding with the exposure (23 days post-ALI), the barrier integrity of the primary NHBEs was assessed and confirmed based on TEER measurements (data not shown). Following 4 hrs. exposure to ABS emissions, no significant changes in TEER were observed between ABS-exposed cells and chamber-control cells or cells maintained in the incubator at the end time points, 0 hrs. or 24h post-treatment (Figure 4.4).

The integrity of the fully differentiated primary NHBEs was further confirmed by visualizing cilia movement (Video 4.1). There were no noticeable differences between the treatments.

In addition, the junctional integrity of the fully differentiated primary NHBEs was assessed using TEM (Figure 4.5). In addition to tight junctions (tj), adherens junctions (aj) and desmosomes (d) were identified, further confirming the presence of junctional complexes and barrier integrity within the NHBEs cultures. Tight-junction and adherens junction formation was also confirmed using immunofluorescence staining of the ZO-1 and E-cadherin proteins (Figure 4.6 and Figure 4.7). There were no noticeable differences between the treatments.

Overall, exposure of NHBEs to ABS emissions did not affect epithelium integrity at the conditions applied.
**Figure 4.2.** Number-based size distribution of cell culture medium (BKGD) and ABS 3-D printer-emitted particles (ABS1 - ABS4) collected in the cell culture medium and analyzed using NTA.
**Figure 4.3.** Representative TEM image of ABS agglomerated printer-emitted particles on the surface of NHBEs cells 24 hrs. after the exposure.
Figure 4.4. Barrier function of fully differentiated NHBEs measured by transepithelial resistance (TEER). Values are means ± standard deviation. N = 2 inserts, n = 2 replicates.
Figure 4.5. Representative TEM images of fully differentiated NHBEs showing junctional complex formation: tight-junctions (TJ), adherens junctions (AJ) and desmosomes (DS).
**Figure 4.6.** Representative labelled immunofluorescence images of fully differentiated NHBEs showing epithelial features, including MUC5AC-producing goblet cells (turquoise) and ZO-1 for tight junctions (green). Nucleus was stained with Hoechst 33342 (blue).
**Figure 4.7.** Representative labelled immunofluorescence images of fully differentiated NHBEs showing epithelial features, including α-tubulin (red) for ciliated cells and e-cadherin for adherens junctions (green). Nucleus was stained with Hoechst 33342 (blue).
4.3.3. Evaluation of NHBEs epithelium differentiation

The bronchial epithelium was composed of fully differentiated airway epithelial cells (Figure 4.8), as noted from H&E and PAS staining. All of the major epithelial cell types, including ciliated respiratory epithelial cells (c), mucous-secreting (goblet cells), and basal cells (b), were identified within the NHBEs layer. This was further confirmed by TEM imaging (Figure 4.9). Ciliated cells and mucous cells were present at the apical side of the epithelium. Histological analysis revealed that overall ciliation did not appear different between ABS-exposed cells and chamber-control cells or cells maintained in the incubator at the end time points, 0 hrs. or 24h post-treatment. Basement membrane and basal cells were present in NHBEs cultures stained with PAS and was seen as a distinct single thin layer at the interface between the epithelium and insert membrane.

The ultrastructure of the NHBEs’ cilia configuration reveals both the cilial basal bodies and the 9 + 2 axoneme arrangements (Figure 4.10).

Immunofluorescence microscopy of the apical surface revealed the presence of mucous (goblet) and ciliated cells detected using the specific markers MUC5AC and α-tubulin, respectively (Figure 4.6 and Figure 4.7).

Overall, exposure of NHBEs to ABS emissions did not affect ciliation or mucus production at the conditions applied.
Figure 4.8. Presentative photomicrographs of ALI epithelium stained with hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) stains showing normal architecture typical of the airway respiratory epithelium (40x magnification). Black arrows point to ciliated respiratory epithelial cells. The yellow box surrounds cells within the basal layer of the epithelium. Asterisks (*) highlight mucus containing goblet cells. Yellow arrows point to the basement membrane separating the epithelium from the underlying filter.
Figure 4.9. Representative TEM images showing the distribution of ciliated (C) and goblet (G) cells at the epithelial surface of fully differentiated NHBEs, and basal (B) cells at the basal region of the epithelium.
Figure 4.10. Representative TEM images of fully differentiated NHBEs showing ultra-structural cilia formation in differentiated NHBEs: (A) longitudinal sections of cilia and basal bodies (arrowheads) and (B) transverse sections of cilia showing 9 + 2 axoneme arrangement (arrowheads).
4.3.4. Evaluation of cytotoxic and inflammatory response in differentiated NHBEs

The viability of cells exposed to ABS emissions was slightly decreased, but it was not significantly different compared with incubator or exposure chamber controls (Figure 4.11A). Cytotoxicity was also evaluated by cell membrane integrity as indicated by LDH leaking from exposed cells. Figure 4.11B showed that exposure to ABS emissions did not induce significant LDH release from exposed cells compared to incubator or chamber controls.

To investigate the inflammatory response from NHBEs cells associated with ABS emissions exposure, screening was performed for a panel of 43 cytokines and chemokines secreted in the basolateral compartment (Table 4.1). At the end of the exposure period (0 hrs.), statistically significant increased levels of IL-7 (1.65-fold) were seen in the cells exposed to ABS emissions compared to the cells maintained in the chamber control environment. At 24 hrs. after the exposure, significant increase in IL-17A (2.35-fold), VEGF (1.59-fold), IFN-γ (2.18-fold), IL-12p70 (1.78-fold), TNF-α (2.10-fold), and MIP-1α (2.70-fold) were also noted in ABS-exposed cells compared to chamber control cells.
Figure 4.11. Cytotoxicity of fully differentiated NHBEs: cell viability (A) and LDH activity (B). Values are means ± standard deviation. N = 3 inserts, n = 4 replicates.
<table>
<thead>
<tr>
<th>Marker</th>
<th>0 hrs.</th>
<th>24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubator</td>
<td>Chamber</td>
</tr>
<tr>
<td>IL-17A</td>
<td>5.20 ±1.61</td>
<td>3.07 ±0.25</td>
</tr>
<tr>
<td>IL-21</td>
<td>0.83 ±0.18</td>
<td>0.56 ±0.15</td>
</tr>
<tr>
<td>IL-22</td>
<td>0.49 ±0.22</td>
<td>0.20 ±0.06</td>
</tr>
<tr>
<td>IL-23</td>
<td>2.85 ±0.88</td>
<td>1.24 ±0.16</td>
</tr>
<tr>
<td>IL-27</td>
<td>69.88 ±26.26</td>
<td>50.73 ±1.65</td>
</tr>
<tr>
<td>IL-31</td>
<td>0.23 ±0.08</td>
<td>0.13 ±0.03</td>
</tr>
<tr>
<td>MIP-3a</td>
<td>1785.57 ±1292.56</td>
<td>79.94 ±14.98</td>
</tr>
<tr>
<td>IL-17A/F</td>
<td>0.15 ±0.18</td>
<td>0.73 ±0.58</td>
</tr>
<tr>
<td>IL-17B</td>
<td>0.19 ±0.19</td>
<td>0.02 ±0.02</td>
</tr>
<tr>
<td>IL-17D</td>
<td>0.10 ±0.19</td>
<td>0.24 ±0.25</td>
</tr>
<tr>
<td>IL-3</td>
<td>5.69 ±3.53</td>
<td>3.53 ±1.6</td>
</tr>
<tr>
<td>IL-9</td>
<td>84.42 ±87.82</td>
<td>69.91 ±5.81</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>IL-23p40</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>21.49 ±</td>
<td>0.40 ±</td>
</tr>
<tr>
<td></td>
<td>38.09</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>IL-4</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>2.75 ± 1.75</td>
<td>3.04 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>0.86 ± 0.39</td>
<td>0.77 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>129.11 ± 165.88</td>
<td>9.09 ± 4.36</td>
</tr>
<tr>
<td></td>
<td>1361.29 ± 872.24</td>
<td>1833.41 ± 35.03</td>
</tr>
<tr>
<td></td>
<td>7.68 ± 5.80</td>
<td>10.84 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>2.75 ± 0.80</td>
<td>1.45 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>7.72 ± 7.14</td>
<td>3.17 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>15849.57 ± 18094.28</td>
<td>7788.59 ± 3445.91</td>
</tr>
<tr>
<td></td>
<td>27.21 ± 17.92</td>
<td>3.26 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>18.62 ± 15.23</td>
<td>14.43 ± 3.17</td>
</tr>
<tr>
<td></td>
<td>3.90 ± 3.47</td>
<td>3.15 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>9.08 ± 7.13</td>
<td>3.89 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>15.33 ± 7.98</td>
<td>10.60 ± 1.50</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>TARC</td>
</tr>
<tr>
<td>-------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Value</td>
<td>4.93 ± 3.19</td>
<td>2.75 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>2.99 ± 1.77</td>
<td>1.76 ± 0.07</td>
</tr>
</tbody>
</table>

Table 4.1. **Cytokines release in basolateral cell culture media.** Values represents mean (pg/ml) ± standard deviation (SD). N = 3 – 5 inserts/treatment/time point, n= 3 replicates. * Statistical Significance at the p < 0.05 level.

a - No SD was calculated, N = 1 insert/time point, n= 2 replicate.
4.4. DISCUSSION

In our previous two studies, we evaluated the toxicity of FFF 3-D printer emissions using both *in vitro* [348] and *in vivo* methods [349]. In the *in vitro* study [348], small airway epithelial cells (SAEC) were exposed in the submerged conditions to 3-D printer emitted-particle freshly collected in the culture medium. In the *in vivo* study [349], male Sprague-Dawley rats were exposed to emissions generated during real-time printing using a whole-body inhalation exposure system. Considering the difficulty of translating toxicological findings obtained in traditional submerged (2D) cell culture models or animal models to human context, this study aimed to evaluate the toxicity of FFF 3-D printing with ABS using a more relevant human ALI organotypic airway tissue model derived from primary normal bronchial epithelial cells and use it as a potential screening tool for other FFF 3-D printer emissions.

Functional characteristics of airway epithelial cells, such as TEER across the epithelial layer [370], ciliary motion [371], and the formation of the junctional complex [351] are essential and key endpoints universally evaluated to study the potential adverse health effects of airborne toxicants or chemicals. Moreover, a change in one of these parameters could be relevant to a variety of respiratory diseases [372].

A previous study [373] showed that ALI cultured airway epithelia from asthma patients display a decreased TEER in comparison to epithelia derived from healthy controls. In this study, the TEER values were lower but not significant in ABS-exposed cells (673 - 680 Ω × cm²) compared to chamber and incubator control cells (691 - 741 Ω × cm²). This finding is intriguing considering that in a case report study [264], a self-employed businessman reported asthma-like symptoms after 10 days working with ABS filament, and his symptoms slowly improved and eventually resolved after he replaced ABS filament with a low emitter filament.

Abnormal ciliary function can lead to inadequate mucociliary clearance, which is associated with various respiratory diseases such as cystic fibrosis, and chronic obstructive pulmonary disease, among others [371]. In this study, light microscopy observation of beating cilia was visualized with a Revolve microscope. However, due to the unavailability of specialized equipment and software, the ciliary beat frequency was
not quantified. However, rigorous observation of two entire inserts per treatment was performed, and vigorous beating of cilia in an apparently coordinated manner was noted as described in other ALI systems [374, 375], demonstrating that differentiated the NHBEs’ have the potential for active mucociliary clearance. However, no noticeable differences were observed between the ABS-exposed cells and the chamber and incubator control cells.

The formation of junctional-complexes (which include tight junctions, adherens junctions, and desmosomes) between neighboring epithelial cells seal off the paracellular space between cells creating a physical barrier against inhaled particulates, pathogens, and other xenobiotics from translocating into the interstitial compartment. The junctional complex also forms a barrier against the movement of inflammatory mediators into the circulation, thereby limiting the activation and recruitment of immune cells to sites of injury [376]. Therefore, the evaluation of junctional-complexes structures presence is an essential feature and important tool used in respiratory toxicity testing.

In the current study, the junctional-complexes were observed and localized by immunofluorescence staining for E-cadherin and ZO-1, and by TEM. To obtain a clear view of the ultrastructural arrangement, ALI cultures were sectioned, followed by TEM imaging. The tight junctions were seen at the apicolateral borders of epithelial cells in the differentiated NHBEs. In close proximity underneath of tight junctions are found adherens junctions, emphasizing their interdependency, and the desmosome, localized more basally. Studies looking at the impact of cigarette smoke, known to contain many types of volatile components and particulates (heterogeneous mixture similar to 3-D printer emissions in regard to its physical state and composition), showed that exposure to cigarette smoke-induced TEER reduction and gene suppression of tight junctions and adherens junction proteins in primary organotypic culture of human bronchial epithelial cells [377], primary small airway epithelial cells [378] or a human airway epithelial cell line (Calu-3) cultured at the air-liquid interface [379]. However, in this study, no noticeable differences were observed between the ABS-exposed cells and the chamber and incubator control cell at the conditions applied, which could be explained by the lower delivered dose in our study.
The integrity of pseudostratified epithelium composed of ciliated and secretory cells and basal stem cells is vital for the process of mucociliary clearance by which multiciliated cells move mucus and trapped pathogens and particles out of the lung [371]. It was found that chronic exposure of primary HNBEs at ALI to cigarette smoke extract impaired the cellular composition of the airway epithelium, reduced the number and the length of ciliated cells, and increased the number of goblet cells [380]. In this study, the major cell types normally associated with in vivo airway epithelium (i.e., ciliated, mucous or goblet, and basal cells) were present within the differentiated NHBEs cultures, and these were replicated in a pseudostratified morphology with ciliated cells interspersed with goblet cells facing the apical side and basal cells spreading along the basolateral membrane. The epithelium exhibited no evidence of major degradation or de-differentiation between any of the treatments applied at the end of time points; there was no noticeable reduction in the number of ciliated cells, no increase in the number of epithelial vacuoles/gaps, and no adverse changes visible by H&E, PAS, and TEM. Ultrastructural analysis of ciliated cells by TEM demonstrated that cilia associated with differentiated HNBEs were of comparable morphology to those of ex vivo tissue, both in terms of the structure of the basal bodies and the 9 + 2 arrangement of axonemes. In the present study, evidence from PAS-stained histological sections and MUC5AC-labelled cultures demonstrated the presence of goblet cells and the production of mucus from differentiated HNBEs. The identification by TEM of mucus globules on the apical surface of HNBEs cultures and goblet cells actively extruding mucus confirmed these findings. Lastly, basal cells were identified from H&E-stained histological sections and TEM images. These cells represent an important component of airway epithelia because they function as progenitor (stem) cells which are involved in repair and regeneration following injury [381].

Cytotoxicity testing is one of the first steps in the evaluation of toxicants. In the current study, the applied treatments (except positive control) had no effect on cellular viability. The LDH release into the media was slightly increased in ABS exposed cells. However, significant increase in IL-12p70, IFN-γ, TNF-α, VEGF, IL-17A, and MIP-1α were observed. Similar findings have been reported previously [382, 383] when it was found that functional changes and oxidative metabolism occurs at concentrations that do not
significantly affect the integrity of the cellular membrane and that the cellular function is typically affected before membrane damage, thus making the cytokines responses a more sensitive measure of cytotoxic effects resulted from exposure to 3-D printer emissions.

The ability of NPs to induce inflammation in tissues is a key factor in determining their toxicity and also a fundamental mechanistic paradigm in nanotoxicology [183, 186]. IL-12p70 is a pro-inflammatory cytokine which promotes induction of Th1 (IFN-γ, TNF-α) and cytotoxic T cell responses [384]. Similarly, in this study, increased levels IL-12p70 was concomitantly with increase in IFN-γ and TNF-α, which are well-known for their role in the regulation of tight junction integrity [385]. TNF-α plays an important role in the caveolin-1-mediated internalization of occludin, which increases epithelial barrier permeability [386]. IFN-γ induces an increase in barrier permeability through the reduction of ZO-1 and occludin expression in an adenosine monophosphate-activated protein kinase (AMPK)-dependent pathway [387]. Therefore, the simultaneous presence of both these cytokines could suggest that exposure to ABS emissions might have a detrimental effect on epithelium barrier integrity through the disassociation of tight junction proteins [388]. Indeed, in this study, the TEER were slightly decrease in cells exposed to ABS emissions but did not reach statistical significance. TNF-α and VEGF have been reported to be involved in the expression and secretion of MUC5AC [389], a major mucin glycoprotein hypersecreted in asthmatic individuals [390, 391]. VEGF is also a potent stimulator of inflammation, airway, and vascular remodeling [392]. MCP-1α was slightly increased in ALI exposed cells. IL-17A, MIP-1α, and MCP-1α increase following exposure to ABS emissions could promote generation of other pro-inflammatory cytokines and chemokines, which might lead to the migration and infiltration of neutrophils and monocytes/macrophages to the inflammation site, as has been previously reported [393-395]. In addition, IL-17A can synergize with other proinflammatory cytokines such as TNF-α and IFN-γ [396], which were found increased in our exposure model as well.

CULTEX® [397-399] and VITROCELL® [400-404] systems are commercially available exposure systems largely used in the field of in vitro inhalation toxicology to test a variety of airborne substances, such as NPs, gases, and complex mixtures. The air-
liquid interface cell exposure (ALICE) system [405-408] and VITROCELL/PARI BOY [409] have been used for aerosolization of NP-containing liquid aerosols. The deposition rates by these devices varied considerably. From the total aerosolized amount in nebulizers, particle deposition ranged between 0.037% for aerosolized polystyrene particles/well in the VITROCELL/PARI BOY system [409], 0.157% in the ALICE system [408], and 2.8% in the optimized ALICE CLOUD system [410]. We also attempted to utilize the VITROCELL® Cloud system in our study; however, due to the difficulty of delivering a reliable dose, we utilize the exposure chamber developed for animal exposure, described in Chapter 3, 3.2.1 paragraph, pages 112-113. Indeed, it has been found that in these systems, the deposition is also particle-dependent [409].

Some advantages regarding the exposure method employed in this study are that 1) the cells were exposed to aerosols and gases via “dry” dispersion, which is comparable to realistic exposure scenarios of the proximal airway exposure experienced by humans, cell-chemical interactions, and faster uptake kinetics of the test articles [411] and 2) the cells were exposed to emissions freshly generated during real-time 3-D printing avoiding the “ageing” effect of long-term storage of NPs as previously reported [412]. With regard to the toxicological effects, overall, the biological effects noted here resulted from the exposure to the complex mixture composed of ultrafine particles and organic compounds.

The purpose of this study was to compare to some extent current findings with outcomes observed under the submerged culture conditions (Chapter 2) or to in vivo study (Chapter 3). However, the number of deposited ABS printer-emitted particles in this study varied largely from $2.2 \times 10^5$ to $3.9 \times 10^7$ ABS particles per cm$^2$ surface area of the insert, making this comparison very challenging. One alternative is to conduct a repeated-dose toxicity study. However, this paradigm could pose challenges as well as it has been determined that performing repeat print runs of the same filament using the same 3-D printer does not yield exactly the same results each time in terms of number of particles or types and amounts of organic compounds [93]. Furthermore, to identify potential threshold values and mechanistic studies, exposure to higher concentrations of ABS emissions has to be performed. Exploring the effect of 3-D printer emissions in co-culture
of epithelial cells with macrophages could provide more accurate insight into the interplay of different cell types at the alveolar barrier.

This short-term exposure pilot study presented here should be considered as preliminary. All results from the study are the findings of one experiment. The currently used exposure chamber has the potential to successfully be used for testing the respiratory toxicity of 3-D printer emissions by ALI approaches. For that, the exposure chamber system requires structural changes to include more sample ports, optimized and validated to ensure a uniform particle deposition throughout the chamber.

4.5. CONCLUSIONS

At the concentration tested, exposure of differentiated NHBEs to ABS emissions does not affect epithelium integrity, ciliation, mucus production, or induce cytotoxicity. However, at the end of 4 hrs. exposure to ABS emissions, we observed increased IL-7 secretion, and at 24 hrs. after the exposure, IL-17A, VEGF, IFN-γ, IL-12p70, TNF-α, and MIP-1α compared to chamber control cells. For the identification of potential threshold values and mechanistic studies, exposure to higher concentrations of ABS emissions has to be performed.
4.6. APPENDIX

Supplemental Figure 4.2. The NTA plate was placed in the exposure chamber during FFF 3-D printing with ABS filament and used for particle collection and characterization, and particle deposition estimation. The cell culture medium (0.5 mL) added the three inserts corresponding to each of the four columns were combined and marked as ABS1 throughout ABS4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean diameter (nm)</th>
<th>Mode diameter (nm)</th>
<th>D50 (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS1</td>
<td>197.9 ± 6.9</td>
<td>164.7 ± 15</td>
<td>186.1 ± 7.2</td>
</tr>
<tr>
<td>ABS2</td>
<td>177.3 ± 2.7</td>
<td>157 ± 3.8</td>
<td>165.2 ± 1.8</td>
</tr>
<tr>
<td>ABS3</td>
<td>150.4 ± 2</td>
<td>136.4 ± 6.2</td>
<td>143.7 ± 2.7</td>
</tr>
<tr>
<td>ABS4</td>
<td>158.9 ± 4.3</td>
<td>130.7 ± 9.8</td>
<td>142.1 ± 4.2</td>
</tr>
</tbody>
</table>

Supplemental Table 4.1. Characterization of ABS particles generated during a 4 hrs. print run: mean hydrodynamic diameter, mode diameter, and size of the particles in the 50th percentile were measured using NTA.
### Supplemental Table 4.2

Calculation of average ABS printer-emitted particle deposition per surface area. First, the concentration of “particles” measured in the cell culture medium (background) was subtracted from the concentrations of the four ABS samples (particle per mL, measured using NTA). These “particles” in the cell culture medium are probably the proteins and other supplemental nutrients added into the ALI complex culture medium to promote cells’ differentiation. Second, to calculate the number of particles per insert, the particle concentration after background subtraction was divided by two to account for the volume of culture medium added per insert (0.5 mL/ 1.12 cm²). Third, to determine the average particle per surface area (cm²), the number of particles per insert obtained previously were normalized per unit cm².

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average particle concentration (particles/mL)</th>
<th>Average particle/mL (after BKGD subtraction)</th>
<th>Average particle/insert (0.5 mL, 1.12 cm²)</th>
<th>Average particle/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium (BKGD)</td>
<td>3.6 × 10⁷ ± 2.5 × 10⁶</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ABS1</td>
<td>7.7 × 10⁷ ± 4.5 × 10⁶</td>
<td>4.1 × 10⁷</td>
<td>2.1 × 10⁷</td>
<td>1.8 × 10⁷</td>
</tr>
<tr>
<td>ABS2</td>
<td>6.0 × 10⁷ ± 3.0 × 10⁶</td>
<td>2.4 × 10⁷</td>
<td>1.2 × 10⁷</td>
<td>1.1 × 10⁷</td>
</tr>
<tr>
<td>ABS3</td>
<td>12.4 × 10⁷ ± 2.6 × 10⁷</td>
<td>8.8 × 10⁷</td>
<td>4.4 × 10⁷</td>
<td>3.9 × 10⁷</td>
</tr>
<tr>
<td>ABS4</td>
<td>3.7 × 10⁷ ± 2 × 10⁶</td>
<td>5.0 × 10⁵</td>
<td>2.5 × 10⁵</td>
<td>2.2 × 10⁵</td>
</tr>
</tbody>
</table>
CHAPTER 5: GENERAL DISCUSSION

Currently, limited understanding is available on the health impact of FFF 3-D printing exposures. The release of particles, particularly in the ultrafine size range, and the complex mixture of toxic VOCs, prompted concerns regarding the possible health implications of 3-D printing emissions. This is important, especially as 1) the use of AM application is expected to increase in industrial workplaces, home-based businesses, and educational settings and that 2) no occupational or consumer exposure limits have been set up yet.

To address the knowledge gaps associated with pulmonary toxicity of FFF 3-D printer emissions, this dissertation presents three studies with the following main goals:

1) To evaluate pulmonary toxicity of FFF 3-D printer emissions in *in vitro* submerged monoculture model.
2) To determine pulmonary and systemic effects in rats resulting from whole-body inhalation exposure to FFF 3-D printer emissions.
3) To study the pulmonary effects of FFF 3-D printer emissions using a relevant human ALI organotypic airway tissue model to mimic the respiratory behavior upon exposure to FFF 3-D printer emissions.

5.1. SUMMARY OF KEY FINDINGS

Initially, a rapid *in vitro* toxicity screening test (Chapter 2) of the two most used thermoplastic filaments in FFF 3-D printing was carried out. This study is the first reported *in vitro* study to evaluate the 3-D printer emission-induced cell toxicity. This first study established that exposure to 3-D printer emissions collected in media induced dose-dependent cytotoxicity in SAEC. Oxidative stress and inflammatory markers (Th1- and Th2-type cytokines) were further investigated to explore the two main nanoparticle-induced toxicity mechanisms, and these analyses were also shown to provoke dose-dependent toxicity. In this study, the dose-responsiveness (regression coefficients) was determined based on the mixed model regression analyses performed on the measured variables using particle number as the independent variable. It is important to emphasize that the cells were exposed to the mixture of particles and organic compounds (styrene,
BPA etc.) trapped in the culture medium during the sample collection. Therefore, it cannot be excluded that the effects measured and their degree of toxicity could be in part due to the specific types of organic compounds released by each individual filament; thus, not solely a result of cell-particle interaction.

The second study (Chapter 3) aimed to evaluate the pulmonary and systemic effects of ABS 3-D printer emissions in a rodent model. This study is the first published in vivo study to evaluate the pulmonary responses from inhalation exposure to 3-D printer emissions. The data indicated that the pulmonary toxic effects caused by exposure to ABS emissions precede systemic toxicity. In detail, at the start of the exposure (day 1), a predominant pro-inflammatory response was seen in BALF represented by an increase in IFN-γ and TNF-α Th1-type cytokines, which was followed by a switch to an anti-inflammatory response by day 15 of exposure represented by a rise in IL-10 Th2-type cytokine. The Th1/Th2 switch could be responsible for the initial “delayed” influx of the alveolar macrophages and its peak occurrence at 15 days of exposure, which corresponded with a significant increase in blood monocytes and platelet counts. Other systemic changes that were noted was that initially (day 1), a significant increase in both hepatic and renal biomarkers (PHOS, AST, LDH) were found, however at day 15 of exposure, only renal biomarkers (PHOS, BUN, URIC) were increased. At the longest exposure duration (day 30), all the endpoints evaluated returned to the control levels. Therefore, at the experimental conditions applied it was concluded that the ABS emissions caused minimal transient pulmonary and systemic toxicity.

The mesentery from the animals used in the respiratory inhalation toxicology studies was shared with our collaborators to evaluate systemic microvascular function [413]. It was found that the ABS emissions significantly reduced arteriolar responsiveness, causing systemic microvascular dysfunction, which was maximum at day 8 of exposure, supporting the hypothesis that the pulmonary exposure to 3-D printer emissions leads to systemic effects. Moreover, microvascular dysfunction can further cause hypertension, diabetes mellitus, diabetic cardiomyopathy, rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis, among others [414, 415].
Due to limitations of submerged cell culture models and challenging data extrapolation from rodents to humans, along with ethical issues raised with animal testing, human-relevant *in vitro* methods have been promoted. Therefore, the third study (Chapter 4) investigated the adverse effects of exposure to 3-D printer emissions using a relevant human ALI organotypic airway tissue model of primary NHBEs. Although the exposure of differentiated NHBEs to ABS emissions did not affect epithelium integrity, ciliation, mucus production, or induced cytotoxicity, a significant increase in cytokines involved in the induction of Th1 responses and regulation of tight junction integrity were significantly increased. Similar to the *in vivo* study (Chapter 3), IFN-γ and TNF-α levels were also increased after ABS emissions exposure. Additionally, the migration of monocytes/macrophages into the lungs observed in *in vivo* study could be explained by increased levels of IL-17A, MIP-1α, and MCP-1α observed in NHBEs exposed cells.

**5.2. OVERALL DISCUSSION AND FUTURE DIRECTIONS**

There are five main strengths of the studies presented here:

1) The toxicological effects of 3-D printer emissions were conducted using both human primary cells and cell lines *in vitro* models in an attempt to identify the integrative biomarkers or biological descriptors for human toxicity prediction as to the ultimate goal.

2) The use of the ALI model, one of the most relevant *in vitro* models of primary human bronchial epithelial cells, after human precision-cut lung slices, which closely mimics *in vivo* and occupational human exposure.

3) The use of *in vitro* human cell models and *in vivo* rat model with the purpose to fill in the translational knowledge gap from animal models to humans.

4) The exposures were conducted using real-time generated 3-D printer emissions, thus avoiding the “ageing” effect of test material or other changes that could be induced by the particle vehicle.

5) The emissions consisted of a relevant mixture of particles and organic compounds, consistent with human occupational exposure.

In the first study (Chapter 2), using a submerged monolayer culture model, the *in vitro* cellular dose or effective dose (particles concentration that reached the cells) was
not determined (this topic is discussed in detail in the next paragraph). However, based on MATLAB calculation, it was estimated that $1 \times 10^7$ ABS particles in suspension are equivalent to 0.626 µg particle mass, or 0.189716 µg/cm$^2$ mass per surface area of well (surface area of well = 0.33 cm$^2$, 0.100 ml of solution/well). This represents the nominal concentration (particles concentration given to the cells) and it was shown to cause a 10% decrease in viability and 20% increase of LDH over control. This particle mass concentration is approximately 60x higher than the lung burden estimated in the in vivo exposure (Chapter 3) at the most extended exposure duration (30 days). Explicitly, the ABS lung burden at 30 days of exposure, as estimated by the MMPD model with clearance (Table 3.3), was 12.4 µg which corresponded to 0.0031 µg/cm$^2$ after normalizing to the surface area of rat lung (4,000 cm$^2$). Therefore, the minimal respiratory and systemic changes observed in the rodent study may be due to lower particle mass deposition than that delivered in the in vitro study, as well as lack of clearance mechanisms.

As mentioned above, the mass of $1 \times 10^7$ particles was estimated to be equivalent to 0.626 µg. In the third study, human airway ALI model (Chapter 4), the particle number per surface area at ALI was calculated for four samples (Supplemental Table 4.2) and it found to be, from the lowest to greatest, $0.022 \times 10^7$, $1.085 \times 10^7$, $1.848 \times 10^7$, and $3.924 \times 10^7$ particle/cm$^2$ which would correspond to $0.013772$ µg/cm$^2$, $0.67921$ µg/cm$^2$, $1.156848$ µg/cm$^2$, and $2.456424$ µg/cm$^2$, respectively, after normalized to the mass of $1 \times 10^7$ particles (0.626 µg). Although the calculated particle mass delivered at ALI is greater than the particle nominal concentration in the submerged monolayer culture model, considering that these results are based on one experiment, more research is needed before making any further comparisons. Thus, the following in vitro to in vivo biological effects comparisons are irrespective of the dose, as dosimetry was the most challenging factor in this project and also acknowledged in the field of nanomaterial risk assessment [416]. This topic and future directions are thoroughly discussed in this chapter for each separate study.

In in vitro studies, evaluating toxicity in submerged monolayer culture (Chapter 2) and human airway ALI models (Chapter 4), the biological effects were assessed at 24 hr.
after exposure. For the ALI model, the biological effects were also measured immediately after the exposure. In \textit{in vivo} studies (Chapter 3), the toxicity was assessed at 24 hr. following repeated exposure (4 hrs./day, 4 days/week) for five duration periods (1, 4, 8, 15, and 30 days). When possible, similar endpoints were selected to compare \textit{in vivo} and \textit{in vitro} toxicity.

In both \textit{in vitro} studies, the cytotoxic effects of 3-D printer emissions were evaluated using similar analytical methods. The emissions collected in the cell culture media used in the submerged cell culture study (Chapter 2) caused more pronounced decreased cellular viability and LDH release than in the ALI exposure (Chapter 4). This could be due to the mucus-producing ability of the differentiated NHBEs, which could have impacted the particle uptake. Ji et al. [417] found that primary bronchial epithelial cells exposed to palladium nanoparticles at ALI showed low uptake in contrast to 1 h after addition to the cultures [418]. However, considering the complexity of 3-D printer emissions, traditional submerged \textit{in vitro} models are unreliable as the addition of gaseous or vaporized constituents to the medium may alter their properties due to interactions and binding with various components of the medium [419]. For this matter, at the moment, a physiologically relevant ALI model combined with an advanced exposure system to ensure accurate dosimetry and uniform delivery is preferred [351].

ROS production and TAC and GPx OS markers were evaluated in the submerged cell culture model (Chapter 2). In the \textit{in vivo} study, MDA and protein carbonyls, known lipid and protein OS markers, respectively, were quantitated (Chapter 3). OS markers were not investigated in the ALI study (Chapter 4). Exposure to 3-D printer emissions is SAEC induced ROS production and decrease antioxidant defense capacity of the cells as seen by decreases in TAC and GPx. However, the levels of MDA and protein carbonyls in the ABS-exposed rats were not statistically significant compared to the air-control animals.

After 3-D printer emissions exposure, in both \textit{in vivo} and \textit{in vitro}, inflammation was the most sensitive parameter for the detection of biological responses. The dysregulation of pro- and anti-inflammatory cytokines and chemokines is a central aspect in the
promotion of inflammation [215, 420]. However, the distinction between pro- and anti-inflammatory effects is not entirely clear mainly due to pathway interactions and their dual pro- and anti-inflammatory effects [215]. Therefore, it is often difficult to use as diagnostic tools.

**Figure 5.1** represents the Venn diagram including the statistically significant cytokines and chemokines increased following exposure to 3-D printer emissions in the SAEC submerged cell culture model, *in vivo* whole-body inhalation exposure, and in the ALI exposure model of differentiated NHBEs. In the *in vitro* submerged culture model, pro-inflammatory cytokines such as IL-16, IL-1β, IL-1α, IL-12p70, IL-13, IL-6, IL-8, and TNF-α significantly increased following exposure to 3-D printer emissions. In the *in vivo* study, IFN-γ and IL-10 were significantly increased in BALF. In the ALI study, IL-12p70, IFN-γ, TNF-α, VEGF, IL-17A, and MIP-1α cytokines were significantly increased in differentiated NHBEs. As seen in the Venn diagram, there was no overlap or relationship between the cytokines measured in all three studies altogether. The first explanation is the time-dependent release of cytokines that were not considered in this study; second, limited commercially available cytokines and chemokines kits for rats; third, additional studies at higher concentrations are needed to establish an exposure-dose-response relationship; fourth, could be due to species differences.

In the first study, among all cytokines, IL-16 increased at the highest slope value compared to other cytokines. IL-16 is a mediator of inflammation by promoting the secretion of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) [421] and also plays a key role in asthma [422]. IL-16 has been shown to play a key role in airway hyper-responsiveness and up-regulation of IgE [423]. In our *in vivo* study, no differences were observed in the IgE levels between the ABS-exposed and air-control animals throughout the entire study. Another cytokine that recently has been found to be involved in asthma pathogenesis is IL-17A [424]. In ALI study, IL-17A increased in the differentiated NHBEs exposed to ABS emissions.
Figure 5.1. Venn diagram showing the statistically significant cytokines and chemokines increased following exposure to 3-D printer emissions in the SAEC submerged cell culture model (Chapter 2), in vivo whole-body inhalation exposure model (Chapter 3) and in the ALI exposure model of differentiated NHBEs (Chapter 4).
The significant increase levels of macrophage inflammatory protein-1, MIP-1α, and slight increase of monocyte chemoattractant protein 1, MCP-1α, in ALI study could justify the migration and infiltration of monocytes/macrophages to the inflammation site observed in in vivo study, as has been previously reported [393, 394, 425]. An increased level of MIP-1α has been detected in BALF from patients with different lung diseases such as allergen-mediated asthma [426], acute respiratory distress syndrome [427], and pulmonary fibrosis [428]. It has been found that MCP-1 stimulates collagen synthesis and production of the pro-fibrotic factor transforming growth factor β (TGF-β) in fibroblasts [429]. MCP-1 levels were significantly elevated in BALF from patients with idiopathic pulmonary fibrosis, interstitial pneumonia with collagen vascular disease, chronic interstitial pneumonia, and sarcoidosis groups compared with normal healthy volunteers [430].

IL-12p70 and TNF-α were found significantly increased in both in vitro studies. TNF-α was also slightly increase in BALF in vivo study at day 1 of exposure. IL-12p70 is a pro-inflammatory cytokine that promotes the induction of TNF-α [381]. TNF-α is a cell-signaling cytokine involved in acute systemic inflammation, the regulation of tight junction integrity [382], and expression and secretion of MUC5AC [389]. TNF-α decreases the protein expression of the tight junction proteins claudin-1, occludin, and ZO-1, as well as to induce cytoskeletal F-actin rearrangement and the mislocalization of occludin and ZO-1 [431, 432]. TNF-α levels were also found increased in the pulmonary sputum of cystic fibrosis patients [433]. In this study, quantification of ZO-1 and MUC5AC following exposure to ABS emissions was not possible, therefore no relationship can be established at this time.

Like TNF-α, IFN-γ was found to disrupt the barrier integrity of epithelial and endothelial cell both in vivo [434] and in vitro [435-439]. Ng et al. [437] suggests that p38 MAP kinase is activated in response to IFN-γ and causes actin rearrangement and altered cell morphology, which in turn mediates endothelial cell hyperpermeability. Smyth et al. [438] suggest that IFNγ stimulates the activity of the Src kinase Fyn, leading to the formation of a complex containing STAT5b, Gab2 and the p85α regulatory subunit of PI3K. The complex assembly results in PI3K activation and a consequent increase in the
macromolecular permeability characteristics of monolayers of the human colon-derived T84 epithelial cell line. In this study, IFN-γ was found significantly increased in the ALI study and in the \textit{in vivo} study, at the beginning of the exposure and decrease thereafter. Disruption of epithelial barrier function triggered by the elevated IFN-γ is accompanied by reduced expression of the TJ proteins, zonula occludens ZO-1 and occluding [435, 439].

In order to properly characterize the correlated risk of 3-D printer emissions exposure as a function of exposure, exposure-dose-response relationships should be established.

In the first study (Chapter 2), a dose-response assessment was completed applying serial dilution method in a neat culture medium. The particles and vapors in chamber air were trapped in the collection medium as the air was drawn through biosamplers's orifices into the collection vessel. The collection medium resulted from two print runs were each delivered at three dilution levels: as undiluted (or 0% dilution), 25% dilution, and 50% dilution, resulting in six doses. Therefore, the concentration ranges obtained for each filament tested were from $1.42 \times 10^6$ to $4.72 \times 10^6$ average particles/cm$^2$ for ABS, and from $1.95 \times 10^7$ to $5.42 \times 10^6$ average particles/cm$^2$ for PC filament. As a note, the particle concentrations used in this study have different ranges for each filament type, with no overlapping concentrations range. Future experiments should be considered, with wider dilution ranges and including the same particle concentrations.

An essential detail in this study that should be considered as a future direction is dosimetry. The average numbers of particles/cm$^2$ used for the dose-response analyses were determined in the suspension and did not represent the dose of 3-D printer-emitted particles that were effectively in contact with the cells during the assay or the internal cellular dose. The determination of the cellular dose is essential for the development of predictive numerical models and reliable \textit{in vitro} screening methods [440]. Therefore, printer-emitted NP-cell interactions studies should be addressed, including NPs sedimentation velocity/flow dynamics, particle density (g/cm$^3$), NP uptake and adsorption to the cell surface, and calculation of delivered dose as a function of exposure time. In general, the impact of exposure to a test article using \textit{in vitro} studies is observed at 24 -
48 hrs. after the particle suspensions are applied to cells. Recently it has been found that estimated delivery of the entire administered dose of engineered SiO$_2$ nanomaterials to cells in culture can take up to hundreds of hours, indicating the importance of test article interactions in physiological fluid, mass transport, and its subsequent toxic effect on cells [441]. The interactions between the 3-D printer particle and the serum proteins contained in culture media are noteworthy. These serum proteins affect the particle size and the mass density due to the presence of the protein corona or of agglomerates which are essential parameters affecting particle transport toward the cell monolayer. However, the determination of the in vitro cellular dose is not trivial, and it can be time-consuming, depending on the method chosen. To evaluate cellular uptake, the most used methods are imaging techniques, such as confocal laser scanning microscopy, TEM, SEM, atomic force microscopy, flow cytometry, inductively coupled plasma-mass spectrometry, live cell imaging, high-content image analysis, X-ray adsorption near-edge spectroscopy [442]. 3-D printer filaments that contain phosphorescent materials that glow in the dark after absorbing light energy are available on the market. These types of filaments could be of interest to pursue particle kinetics of deposition and transport studies.

In the second study (Chapter 3), the animals were exposed to a single concentration which represented the maximum concentration obtained during simultaneously printing of three desktop printers. With respect to inhalation as the main route of exposure, a minimum of three exposure concentrations plus air-exposed controls should be used. To advance this study, one alternative would be to modify the emissions generation chamber to accommodate the installation of additional 3-D printers or printer nozzles ports, and the second alternative would be to conduct nose-only exposures able to provide reliable, uniform, and efficient aerosol delivery. The retained dose (lung burden) at the end of exposure is essential for the evaluation of the results of inhalation studies and deemed as a requirement by the OECD revised Test Guideline 413 [422] for subchronic rodent inhalation in order to provide exposure-dose-response relationships data for quantitative inhalation risk assessments. In this study, the particle deposition mass for the head/nose region, tracheobronchial region, alveolar regions were estimated using the MPPD model. At 30 days the alveolar deposition estimates were about 25 µg and 12 µg, representing the model without and with clearance, respectively, which is
about 30 times and 60 times lower, than in the first *in vitro* study (Chapter 2). Based on the *in vivo* minimal effects, this single concentration tested here could be at most classified at LOAELs. However, additional studies are needed to obtain a complete hazard ranking, establishing no observed adverse effect levels (NOAELs) and confirm LOAELs. Ideally, a positive and/or negative benchmark material should be selected, against which the 3-D printer emissions can be compared. For example, laser printer emissions which have been thoroughly tested and are similar to 3-D printer emissions in the sense that the laser printers are known to generate particles and organic compounds, however, these emissions also release fibers and inorganic gases [443].

The availability of robust lung burden data from *in vivo* inhalation studies is important for the dosimetric integration of *in vivo* and *in vitro* responses based on *in vivo* to *in vitro* dose extrapolations (IVIVE) methods [444] which involves normalization by the metric “per cell surface area” or “per cell.”

Ma-Hock et al. [444] proposed the following six steps for *in vitro* dose setting (OBIIV) based on nanomaterial *in vivo* organ burden data which could be applied to further explore the toxicological effects of 3-D printer emitted-particles:

1) Determine *in vivo* scenario to be reflected *in vitro* by establishing the target organ (the lung), route of exposure (inhalation), and which *in vivo* exposure duration will be reflected (short-term or long-term exposures).

2) Identify *in vivo* organ burden at (or close to) LOAEC. If *in vivo* organ burden data are not available, the next steps can be taken: 1) establish LOAEC experimentally (measured), and 2) model lung burden using the MPPD model to estimate the relative *in vivo* particle deposition in the airways at the LOAEC (the use of MPPD estimations adds a further uncertainty in the order of a factor of two to the IVIVE, Step 6).

3) Extrapolate *in vitro* dose from *in vivo* organ burden. At this step, the *in vivo* organ burden is extrapolated to *in vitro* effective dose (particles concentration that reached the cells) by calculating 1) the lung burden per alveolar surface area, which would represent the *in vitro* dose expressed as particle mass per culture well surface area (µg/cm²) or particle surface area per culture well surface area.
(cm²/cm²), and 2) the lung burden per cell dose which would represent the in vitro dose expressed as particle mass per single cultured cell (pg/cell).

4) Extrapolate in vitro effective dose to in vitro nominal concentration (particles concentration given to the cells).

At this step, the type of cell exposure model that will be applied is taken into account. For example, in the case of the aerosol exposure of cells at ALI, the determination of nominal concentration to effective dose is more straightforward, as it can be controlled by equipment. If submerged exposure is used, the nominal concentration that is correlated to the effective dose must be determined using models such as NANOGENOTOX protocol, DG, or ISDD modeling, which are considering particle’s sedimentation and diffusion. However, because the 3-D printer particle’s sedimentation and diffusion properties are still unknown, as discussed in this chapter, in this situation, the in vitro effective dose to in vitro nominal concentration should be determined experimentally using imaging techniques, as presented above in the paragraph that discusses the dosimetry challenges involving 3-D printer emitted-particles.

5) Set in vitro dose range around LOAEC-equivalent dose to allow establishing a dose-response relationship. The dose range should be at least two orders of magnitude to account for uncertainties.

6) Consider variability and uncertainties related to the organ burden data and the in vitro test system specificity potentially affecting in vitro interpretation. For example, Ma-Hock et al. [444] determined that lung burden and alveolar surface area measurements have <15% and 20 - 25% uncertainties, respectively. Also, the uncertainty for the alveolar epithelial type I or II cells (AET-I, AET-II) were approximately 12% and 7.5%, and can be accounted for using the pg/AET-I or pg/AET-II dose metrics [444]. It is essential to identify the in vitro test system parameters that can affect the interpretation of measurements, such as particle properties that are affected by the surrounding environment (in the lung lining fluid, in vivo, versus different culture media, in vitro), possible interference of particle with in vitro endpoint detection methods, and exposure duration and time dependency of in vivo versus in vitro effects.

The development of a human 3D airway epithelial tissue-like culture system that can be maintained in vitro is significant progress in the field of environmental air pollutant
risk assessment. One significant experimental advantage is that these cultured primary cells can be directly exposed to aerosols at the ALI, simulating the relevant physiologically conditions of human or in vivo exposure. In the third study (Chapter 4), the ABS printer-emitted particle deposition per surface area of inserts in the ALI study varied greatly from $0.022 \times 10^7$ to $3.924 \times 10^7$ average particle/cm$^2$, which confirms the challenges related to the aerosol dosimetry. However, it should also be emphasized that this study represents a preliminary study in which only one experiment was performed. Therefore, additional studies are needed for the identification of potential threshold values and mechanistic studies. Especially important are the deposition efficiency and uniformity of deposition at ALI. Oldham et al. [445], using Vitrocell® 24/48 ALI exposure system, which is able to directly expose each insert through individual aerosol inlets, found that the deposition efficiency decreases with particle size. For example, polystyrene monodisperse solid fluorescent particles with mass median aerodynamic diameters of 1.1 µm and 0.51 µm had a deposition efficiency of 0.06 and 0.013%, respectively. Next, characterization of the 3-D printer emissions at ALI are needed, as the aerosol delivered to the cell surface may be different from the generated (parent) aerosol. Since the 3-D printing emitted particles are mainly composed of semi-volatile organic compounds, if heating and dilution of the aerosol with humidified air is used (to maintain the exposure chamber at 37 °C), attention should be given to the particle to gas phase change of semi-volatile chemical constituents.

The EDX analysis of particles collected in the culture media showed the presence of Ni, Cr, and Fe, metals associated with health and environmental concerns [446]. Therefore, to further our understanding of the 3-D printer emitted particle’s toxic effects, comprehensive solubility/dissolution studies should be considered. Dissolution affects particle biopersistence and biokinetics, including retention [416]. Two such methods can be applied by 1) determining the equilibrium (or quasi-dynamic) solubility (µg/L) and 2) determining dissolution rates (ng/cm$^2$/day dissolved).

The first method is characteristic of a static system reflecting in vitro conditions, which involves suspending particles in a medium, incubation, removal of remaining solids by centrifugation or ultrafiltration, and measurement of the test analyte in solution [447].
The second method is characteristic of a dynamic (non-equilibrium) system and mimics more closely the \textit{in vivo} situation, that uses culture media composition that simulates extra- and intracellular lung environments, with both fluid phase and solid phase product analysis. In these systems, dissolved ions pass through a membrane with a pore size that excludes the test particles and are continually removed from the system using a “push through” (ultrafiltration) or “double chamber” (macrodialysis), thus achieving non-equilibrium conditions over time course of the study [416]. The dialysate is collected in separate volumes. The target analyte is quantitated in the collected fractions and the waste that was not sampled. Because a large number of 3-D printer emitted particles are in the nano-size range, an appropriate separation membrane should be carefully considered in this situation.

The composition of the dissolution fluid is another critical factor with respect to closely simulating \textit{in vivo} conditions, e.g., intracellular conditions (pH \textasciitilde 4.5) or extracellular conditions (pH \textasciitilde 7.4). The extracellular fluid determines the dissolution of inhaled particles in the epithelial lining fluid. The intracellular fluid measures dissolution in the phagolysosome of alveolar macrophages following phagocytosis of the deposited particles [448]. Additionally, Stefaniak et al. [449] found that the dissolution rates can be influenced by the variations in phagolysosomal fluid simulant composition and emphasized the importance of a fluid simulant to mimic the chemical properties of the fluid in the biological environment in which they are present.

Dynamic dissolution studies could be of interest pursue in the future for testing the particles emitted from various thermoplastic filaments. The dissolution rates could provide valuable information about their biopersistence, which will allow prediction of the overall pulmonary clearance rate and associated overall retention halftime of particles of different solubilities [450]. These results may be further considered as a grouping tool into high, moderate, low, and insoluble particles or used as inputs into the MPPD model for purposes of human risk extrapolation modeling. However, this work will require validation and standardization. Also, the transformations of shape and size distribution of the residual solids after testing can be further evaluated using electron microscopy methods, supported by EDX.
Although biocompatibility evaluation of the 3-D printed parts was not the focus of this work, it is worthwhile to mention some research findings in this area as it might apply to future studies. Because the use of 3-D printed parts as medical and dental devices is a growing industry [451], biocompatibility studies of printed parts are currently being undertaken [128, 452]. These studies focus on extractables that could cause allergic, inflammatory, or other toxicological reactions in patients. Extractables are solvent or thermal extracted chemical compounds that can be pulled from the primary product under controlled laboratory conditions.

Nahan et al. [128] conducted a screening for volatile, semi-volatile, and non-volatile extractables compounds in FFF ABS orthopedic casts and found that these extractables included potentially-genotoxic isomers of the styrene-acrylonitrile trimer. Rindelaub et al. [452] investigated the extractables profiles from FFF 3-D printed medical devices using PC, PETG, PLA, and a PLA polymer advertised as “FDA-approved”. The material marked as “FDA-approved”, corresponds to the use of an FDA compliant resin (as per the manufacturer) and does not actually hold U.S. Food and Drug Administration approval. It was found that the FDA-approved PLA polymer extracts had the greatest concentrations of semi-volatile organics, which included L-lactide and phthalate plasticizer (DEHP), and particulate matter, raising caution about the potential human hazard of these extractables. Animal studies [453, 454] show that DEHP causes reproductive and developmental toxicity. This could be in line with the results of Oskui et al. [271], who studied the toxicity of STL and FFF ABS 3-D printed parts on the Zebrafish embryo and found that exposure to parts from both types of printers caused hatching and developmental abnormalities, with STL-printed parts being significantly more toxic than FFF-printed parts. However, in this study, the extractable or leachable were not measured. De Almeida et al. [272] also studied the toxicity of commonly used 3-D printing polymers on bovine embryos and found toxic effects of the polymers on embryo development. DEHP and polyethylene glycol were detected in the cell culture media. Rindelaub et al. [452] also reported that annealing of FDA-approved PLA 3-D printed medical devices reduced the extractable semi-volatile organic compound concentrations by 43%, and particulate matter concentrations by a factor of 50.
Overall, toxicological evaluation of FFF 3-D printer emissions was conducted in three different conditions, 1) traditional submerged culture of human small airway epithelial cells, 2) repeated whole-body inhalation exposure of rats to freshly generated aerosols, and 3) advanced human ALI organotypic airway tissue model derived from primary normal bronchial epithelial cells. At the experimental conditions applied, in both in vitro conventional model and in vivo, 3-D printer emissions produced minimal to moderate pulmonary toxicity. Furthermore, these findings were consistent with results observed in the physiologically relevant in vivo-like in vitro model cultured at ALI. In conclusion, these studies indicate that the FFF 3-D printer emissions could induce moderate toxicological effects. These studies are significant as they are amongst the first and comprehensive studies published to evaluate the pulmonary and systemic toxicity of 3-D printer emissions. Further studies are needed to establish a more comprehensive exposure-dose-response relationships and integration of in vivo and in vitro responses.

This work is relevant to public health because it will help to develop and implement evidence-based exposure limits and control strategies, as currently, no exposure limits exist, and thus impact the health of 3-D printer users in both industrial and non-industrial settings.
REFERENCES


79. all3dp.com, Buyer’s Guides. 2020.


Xu, Y., et al., *Effects of diesel exposure on lung function and inflammation biomarkers from airway and peripheral blood of healthy volunteers in a chamber study*. Particle and Fibre Toxicology, 2013. 10(1): p. 60.


352. Bogdanffy, M.S. and D.A. Keller, Metabolism of xenobiotics by the respiratory tract. Toxicology of the Lung, 1999. 3.


Toxicology and 3D EpiAirway models for toxicological and e
Fields, W., et al., Assessing the respiratory toxicity of dihydroxyacetone using an in vitro human
Wang, Y., et al., Use of the Cultex® Radial Flow System as an in vitro exposure method to
Steinritz, D., et al., Characterization and application of the VITROCELL VC1 smoke exposure system


SUMMARY

- Highly organized and expert on evaluating pulmonary toxicity using *in vitro* (submerged, co-culture, and air-liquid-interface) and *in vivo* (rodents) models
- Analyzed and evaluated the risk associated with exposure to diesel/biodiesel emissions, crystalline nanocellulose, asbestos/asbestiform fibers, carbon nanotubes, laser printer particles, surgical smoke, and 3-D printer emissions
- First-author of 3 peer-reviewed publications and co-author of 10 publications
- Seeking a position in toxicology and human health risk assessment

EDUCATION

<table>
<thead>
<tr>
<th>Year</th>
<th>Degree</th>
<th>Institution</th>
<th>Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017 – 2021</td>
<td>Ph.D. Pharmacological and Pharmaceutical Sciences</td>
<td>West Virginia University, School of Pharmacy</td>
<td>Toxicology</td>
</tr>
<tr>
<td></td>
<td>Dissertation: “<em>In vitro</em> and <em>in vivo</em> toxicological evaluation of emissions from the fused filament fabrication three-dimensional printing.”</td>
<td>Advisor: Yong Qian, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>2009 – 2012</td>
<td>M.S. Applied and Environmental Microbiology</td>
<td>West Virginia University, College of Agriculture, Natural Resources &amp; Design</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thesis: “The growth of <em>Chlorella vulgaris</em> and <em>Chlamydomonas reinhardtii</em> for biodiesel production and carbon dioxide capture.”</td>
<td>Advisor: Alan Sexstone, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>2004 – 2006</td>
<td>M.S. Applied Enzymology</td>
<td>University of Bucharest, College of Chemistry, Department of Biochemistry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Advisor: Serban Fleschin, Ph.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000 – 2004</td>
<td>B.S. Chemistry</td>
<td>University of Bucharest, College of Chemistry, Department of Biochemistry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration: Technological Biochemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Project: “A polyphasic study on the taxonomic position of natural yeast isolates involved in winemaking.”</td>
<td>Advisor: Tatiana Elisabeta Vassu-Dimov, Ph.D.</td>
<td></td>
</tr>
</tbody>
</table>

RESEARCH EXPERIENCE

07/02/2012 - Present
Centers for Disease Control and Prevention (CDC)
National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV
Position: Regular fellow/ORISE fellow
• Designed, planned, and carried out experiments in order to investigate the adverse effects of exposure to acrylonitrile butadiene styrene (ABS) and polycarbonate (PC) emissions generated from 3-D printers using submerged culture of human small airways epithelial cells (SAEC).
  o Established that emissions released by both thermoplastic filaments induced toxicological effects as seen by a dose dependent significant cytotoxicity, oxidative stress, cell death, and production of pro-inflammatory markers.
  o Measured variables: cell viability, lactate dehydrogenase activity (LDH), ROS production, glutathione peroxidase, total antioxidant capacity, apoptosis, necrosis, and several pro- and anti-inflammatory cytokines and chemokines.
  o Determined dose-response relationship between the measured variable and the numbers of the 3-D printer emitted particles based on mixed model regression analyses (JMP).

• Determined the subchronic inhalation respiratory and systemic toxicity of ABS and PC filament emissions in rats based on procedures described in OECD and EPA Guidelines, and National Toxicology Program studies.
  o Established that the emissions from ABS and PC filament caused minimal transient pulmonary and systemic toxicity, suggesting that the emissions elicited an adaptive response in this exposure paradigm.
  o Measured pulmonary injury, inflammation, and oxidative stress as well as markers of systemic toxicity.
  o Endpoints measured:
    ▪ Oxidative stress markers in lung homogenates: malondialdehyde and protein carbonyls.
    ▪ Systemic toxicity: complete blood count, serum chemistry analysis (liver, kidney, and muscle injury markers).
    ▪ Histopathological changes of the lung, and nasal passages.

• Designed, planned, and carried out experiments in order to investigate the adverse effects of exposure to ABS emissions using primary human bronchial epithelial cells (NHBE) grown at air-liquid interface.
  o The goal of this study was to recapitulate the in vivo inhalation exposure responses and provide robust data for quantitative inhalation risk assessments for use in a regulatory context of other types of 3-D printer filaments, as alternatives to animal testing.
  o Assessed the morphological changes of HBEpC 3D culture characteristics and integrity, based on H&E and periodic acid-Schiff (PAS) staining, immunofluorescence for α-tubulin, E-cadherin, MUC5AC, and ZO-1, trans-epithelial electrical resistance (TEER), and cilia beating.
  o Evaluated cytotoxicity, oxidative stress, tissue injury/repair, and inflammatory and immune system regulation markers.
Other research projects involved outside my dissertation work:

- Performed *in vitro* and *in vivo* research to investigate the pulmonary toxic effects of diesel/biodiesel emissions, crystalline nanocellulose, asbestos/asbestiform fibers, carbon nanotubes, laser printer particles, and surgical smoke.
- Participated in hypothesis driven conceptual design of research direction.
- Assisted in data compilation, data review, data interpretation, and manuscripts preparation.
- Optimized and performed assays to determine various experimental endpoints.

Managerial responsibilities:

- Authored Animal Use Protocols (Topaz Enterprise system) complying with the regulations and guidelines set forth by the Institutional Animal Care and Use Committee (IACUC).
- Amended the Animal Use Protocol to include microvascular, neurotoxicity, nephrotoxicity, and reproductive toxicity studies of the 3-D printer emissions in collaborations with three other groups of researchers.
- Coordinated and supervised the animals' conditions and procedures while hosted by the Animal Facility and the Inhalation Facility.
- Assigned and tracked specimens and samples generated during the experiments as per CDC Specimen and Sample Management policy.
- Developed and implemented laboratory SOPs.
- Oversaw all equipment, performed weekly equipment calibration and preventive maintenance, inventory, and ordering of reagents and supplies.
- Trained and supervised the effective working of six new employees.
- Prepared “sole source” procurement documentation for purchasing equipment, technology, or services.

01/12/2009 – 05/11/2012
West Virginia University, Davis College, Morgantown, WV
Position: Graduate research assistant
Advisor: Alan Sexstone, Ph.D.

- Developed a growth method of two strains of green microalgae, *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (UTEX 90), to increase CO2 capture and biodiesel production.
- Tested the effect of different culture media, pH, light intensities, and CO2 concentrations on *C. vulgaris* and *C. reinhardtii* growth.
- Initiated and maintained a culture collection of algae isolated from Morgantown, WV surroundings areas.
- Prepared materials for scientific tests and collected experimental data.
- Analyzed data in JMP and maintained accurate experimental records.
RESEARCH SKILLS

Molecular Biology
- Transcriptomics: DNA and RNA extraction and purification from cells and tissue, DNA and RNA quantitation.
- Proteomics: Protein extraction and purification from cells and tissues, protein quantitation, gel-electrophoresis, western blotting, ELISA.
- Cloning: Bacterial transformation, plasmid purification, PCR.

Tissue Culture
- Primary cell and immortalized cell line maintenance, aseptic technique, cryopreservation, in vitro exposures (monoculture, co-culture, air-liquid interface).
- Cell-based assays: MTT proliferation assay, AlamarBlue cell viability assay, DCFDA Intracellular ROS assay, multiplexing, high content analysis.

Microscopy
- Immunofluorescent microscopy of fixed cells and FFPE tissue.

Flow cytometry
- Tissue digestion, antibody labeling, FACS analysis.

Animal procedures (mice and rats)
- Handle and restrain, weigh animals, anesthesia, euthanasia.
- Pharyngeal exposure.
- Collect blood and tissues (lung, liver, spleen, kidney, heart, brain, small intestine, seminal vesicles, testes).
- Collect bronchoalveolar lavage, and cytologic analysis.
- Intraperitoneal and tail injection.

OTHER PROFESSIONAL EXPERIENCE

08/01/2004-01/10/2008
S.C. Ciprod-Pharm S.R.L., Bucharest, Romania
Position: Biochemist & Internal auditor
- Prepared documents and obtained approval for good manufacturing practice in manufacturing, packing, labeling, or holding operations based on European Union recommendations for over 100 dietary supplements and nutraceuticals.
- Analyzed microbiological and physicochemical properties of medicinal plants used in manufacturing, according to the European Pharmacopoeia.
- Conducted on-site independent evaluations to ensure that Good Manufacturing Practice and Quality Management System are adequately implemented.
- Prepare formal written reports setting forth recommendations for local and corporate management to strengthen and improve operations.
- Provided audit support for external audits and miscellaneous enhancement initiatives as needed.
- **PEER-REVIEWED JOURNAL ARTICLES**


**SUBMITTED JOURNAL ARTICLES**


**JOURNAL ARTICLES IN PREPARATION**

2. Krajnak, K., Farcas, M.T., McKinney, W., Waugh, S., Mandler, K., Jackson, M., Matheson, J., Thomas, T., Qian, Y. Inhalation of polycarbonate emissions generated during 3D printing processes induces expression of factors indicative of injury in the central nervous system.

3. Krajnak K., Farcas, M.T., McKinney, W., Waugh, S., Mandler, K., Jackson, M., Matheson, J., Thomas, T., Qian, Y. Inhalation of polycarbonate emissions generated during 3D printing processes induce changes in neuroendocrine function.
**ORAL PRESENTATIONS**


**POSTER PRESENTATIONS**


**AWARDS & FELLOWSHIPS**

<table>
<thead>
<tr>
<th>Year</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2021</td>
<td>Graduate Student Excellence Award from International Society of Regulatory Toxicology and Pharmacology / SOT Regulatory and Safety Evaluation Specialty Section</td>
</tr>
<tr>
<td>2021</td>
<td>Best Applied science research poster from CDC’s 2021 Laboratory Science Symposium</td>
</tr>
<tr>
<td>2021</td>
<td>Outstanding Graduate Student. Nanoscience and Advanced Materials Specialty Section.</td>
</tr>
<tr>
<td>2021</td>
<td>Robert &amp; Stephany Ruffolo Pharmacy Graduate Fellowship from WVU</td>
</tr>
<tr>
<td>2019 – 2021</td>
<td>Oak Ridge Institute for Science and Education (ORISE) fellowship</td>
</tr>
<tr>
<td>2020</td>
<td>Stitzel Graduate Student Support for Pharmacy. School of Pharmacy, WVU</td>
</tr>
<tr>
<td>2020</td>
<td>Occupational and Public Specialty Section Student travel award</td>
</tr>
<tr>
<td>2020</td>
<td>Allegheny-Erie Society of Toxicology Student Travel Award</td>
</tr>
<tr>
<td>2020</td>
<td>School of Pharmacy, WVU. Graduate Student Travel Support</td>
</tr>
<tr>
<td>2020</td>
<td>iTox Working Group, WVU. Graduate Student Travel Award</td>
</tr>
<tr>
<td>2019</td>
<td>Society of Toxicology. Graduate Student Travel Award</td>
</tr>
<tr>
<td>2019</td>
<td>Toxicology Working Group, WVU. Graduate Student Travel Award</td>
</tr>
<tr>
<td>2019</td>
<td>Office of research and graduate education, WVU. Student Travel Award</td>
</tr>
<tr>
<td>2019</td>
<td>School of Pharmacy, WVU. Graduate Student Travel Support</td>
</tr>
<tr>
<td>2018</td>
<td>Nanomedicine, and Nanotoxicology, Pharmaceutical Sciences Research Symposium, WVU. Best poster presentation- 2nd place.</td>
</tr>
</tbody>
</table>
2016  **Charles C. Shephard Science Award**: co-author of an outstanding paper nominee.


2012  Student Memorial Scholarship Award, WVU

2012  Excellence in Civic Engagement Award, WVU

**PROFESSIONAL AFFILIATIONS**

<table>
<thead>
<tr>
<th>Year</th>
<th>Affiliation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013 – Present</td>
<td>Society of Toxicology - Student member</td>
</tr>
<tr>
<td>2017 – Present</td>
<td>Allegheny-Erie Society of Toxicology Regional Chapter – Student member</td>
</tr>
<tr>
<td>2017 – Present</td>
<td>Women in Toxicology Special Interest Group – Student member</td>
</tr>
<tr>
<td>2017 – Present</td>
<td>Nanoscience and Advanced Materials Specialty Section – Student member</td>
</tr>
<tr>
<td>2017 – Present</td>
<td>Occupational and Public Specialty Section – Student member</td>
</tr>
<tr>
<td>2019 – Present</td>
<td>Inhalation and Respiratory Specialty Section – Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td>Exposure Specialty Section – Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td>American College of Toxicology - Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td><em>In vitro</em> Specialty Section – Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td>Regulatory and Safety Evaluation Specialty Section – Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td>Risk Assessment Specialty Section – Student member</td>
</tr>
<tr>
<td>2019 – Present</td>
<td>Phi Lambda Sigma - The Pharmacy Leadership Society – Student member</td>
</tr>
<tr>
<td>2020 – Present</td>
<td>Rho Chi - The academic honor society in pharmacy – Student member</td>
</tr>
</tbody>
</table>

**PROFESSIONAL SERVICE**

<table>
<thead>
<tr>
<th>Year</th>
<th>Service</th>
</tr>
</thead>
<tbody>
<tr>
<td>2020 – 2021</td>
<td>Vice-student representative Nanoscience and Advanced Materials Specialty Section</td>
</tr>
<tr>
<td>2020 – 2021</td>
<td>Graduate Student Leadership Committee Programming Subcommittee</td>
</tr>
<tr>
<td>2020 – 2021</td>
<td>Graduate Student Leadership Committee, Nanotoxicology</td>
</tr>
<tr>
<td>2020</td>
<td>NIOSH Internal reviewer for SOP</td>
</tr>
</tbody>
</table>

231
2019  Served as a Continuing Education course volunteer at the 2019 Society of Toxicology Annual Meeting in Baltimore, MD.

2019 – 2020  Peer-Reviewed articles for Ecotoxicology and Environmental Safety, Toxicology and Applied Pharmacology, and Reproductive Biology journals

2012 – 2018  Trained four NIOSH graduate students in various *in vivo* and *in vitro* techniques

2011 – 2012  Trained and supervised the work of three WVU undergraduate students in microbiology techniques