ABSTRACT

NEEDHAM, ERINN CHRISTINE. Atomic Layer Deposition for the Modification and Creation of Nanomaterials. (Under the direction of Dr. Gregory N. Parsons).

Atomic layer deposition (ALD) is a vapor-phase technique for the conformal deposition of material with sub-nanometer precision, making it an ideal process for modifying and even creating nanomaterials. The focus of this dissertation is the study of how ALD precursors interact with organic materials, namely polymers, to create selectively deposited nano-scale patterns and how ALD coatings modify biological responses to nanomaterials, namely carbon nanotubes (CNT), after inhalation.

Nanoscale patterning is vital to the semiconductor industry. With features becoming smaller and more complex with each passing year, new techniques are required to meet the needs of the industry. The ability to selectively pattern a material onto a wafer is of particular interest for the replacement of costly etching steps.

In the first half of this dissertation, a method for the selective deposition of nano-scale patterns is presented. Patterned polymers were used as sacrificial sponges to soak up ALD precursors for the creation of metal-oxide features. Meanwhile, deposition in areas without polymer was limited to the monolayer regime. Following infiltration, the saturated polymer was burned away and the precursor oxidized to form a metal oxide reproduction of the polymer pattern.

Determining the reaction between the ALD precursor, trimethylaluminum, and polymer, poly(methyl methacrylate), helped to achieve patterning by informing the proper selection of reactor temperature as well as exposure and purge times. Using this technique, features from tens of nanometers to tens of microns were patterned uniformly and simultaneously across a 150 mm wafer.

Finally, this technique was extended to pattern two different materials using only one patterned polymer layer. ALD was first used to deposit a metal oxide where there was no polymer. By selecting ALD precursors that do not react within or on top of the polymer, selective deposition of the first material was achieved. Following this, the polymer was infiltrated as before to
selectively deposit the second material. By patterning two materials from one patterned polymer, no pattern alignment between materials is necessary.

The reaction mechanism determined for this system can be applied and expanded to other vapor-phase metal-organic interactions with polymers. The ability to make and align nanoscale features is critically important for manufacturing improved semiconductor devices.

The second half of this dissertation focuses on how modification of CNT affects biological response in a material-dependent manner. CNT have unique physical and chemical properties that lead to applications in many areas including: electronics, high-strength materials, filtration and drug delivery. By surface-modifying these materials, a whole new realm of applications appears.

Despite the benefits these coatings may provide (e.g., photocatalytic properties and increased conductivity) they can also alter the toxicological response to MWCNT. In rodent models, the inhalation of MWCNT can lead to inflammation and fibrosis. Here, we observed that ZnO coatings on MWCNT led to an acute inflammatory response but did not change the fibrotic response in mice following inhalation.

The contribution of ZnO coating dissolution was still unknown following the *in vivo* study with mice. Alumina, ZnO and aluminum-doped ZnO (AZO) coatings on MWCNT were studied *in vitro* using various cell lines to determine the contribution of ions to toxicity. AZO is less soluble than ZnO and composed only of previously-characterized materials.

We discovered that the concentration of Zn$^{2+}$ in solution correlated with levels of cytotoxicity *in vitro* and differences in dissolution between AZO and ZnO coatings led to differences in pro-inflammatory cytokine release. This knowledge can assist with the toxicological assessment of other pure and composite nanomaterials and lead to the creation of safer nanomaterials.
Atomic Layer Deposition for the Modification and Creation of Nanomaterials

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

To all those that came before me, without whom the tools necessary to complete this would not exist.

To my teachers, both in and out of school, for my understanding of the world around me.

To my parents for always answering my questions, teaching me to solve problems, and having the patience to do the first two.

To my brother for being the arts to my crafts and my partner in crime.

And finally, to my silly husband for keeping me sane.
BIOGRAPHY

Erinn Christine was born to Sean and Jayne Dandley; two wonderfully weird, supportive and generally bad ass parents; in 1989. She has one brother, Michael, who has been taller and more patient than her since he was two and she was four. Throughout her life she has picked up many random skills from a diverse cast of characters that have led her to being able to complete this body of work. The Dandley’s are quite hands on problem solvers that aren’t afraid to get dirty. From a young age Erinn would help out with projects around the house with the aid of her parents. By middle school she could solve most problems with a roll of duct tape.

Stubborn and fiercely independent she sometimes needed guidance from one brave soul or another. In one such instance it was gently suggested to her that she join track and field in high school. After deciding she hated running she specialized in short sprints, javelin and pole vault. Her pole vaulting career continued at the University of Massachusetts-Amherst where both her parents and her brother went to college. Pole vaulting introduced her to some of her best longtime friends and travel companions.

Throughout school Erinn showed a propensity for math and science. Challenged with a tough chemistry teacher in high school she learned a serious amount of material on the subject. This led her father to suggest a major in chemical engineering at UMass after a year of entrepreneurial studies.

The summer of 2010 Erinn went to the University of Kentucky to do research for the first time. There she not only fell in love with research but also her husband, Craig Needham. She went on to do research under Shelly Peyton at UMass for the remainder of her time in undergrad.

In the fall of 2011 she tore her ACL while pretending she was good at balancing and fell off a slack line. She promptly gave herself tendonitis in her ankle from hopping around lab trying to do research on one leg. This really highlighted how much she would miss research if she took a job in industry right away and led her to apply to graduate school.
After searching far and wide for the school that fit her best, remember she is stubborn, she decided on NC State, which was really great because Craig was already there studying for his PhD. There she pursued her PhD in chemical and biomolecular engineering under Dr. Greg Parsons. She was also fortunate to be co-advised by Dr. Bonner in toxicology and Dr. Chang in mechanical engineering.

In 2015, before completing their PhDs, Craig and Erinn were married surrounded by many goats along with their friends and family. Up next they are moving to Portland, OR to see what the west coast has to offer.
ACKNOWLEDGMENTS

I sincerely thank my parents for creating me as well as teaching me to fix things, question “known” answers, and be an independent person in the world. I thank my brother for forgiving me for being the oldest and for making the world a more beautiful place. I also thank all of my new Needham family for their support and encouragement as well as making me feel so welcome.

My advisor, Greg Parsons, I would like to thank for his encouragement to follow hunches, be creative, explore how things work, and collaborate with people with different specialties. Through his guidance I have greatly increased my research proficiency. I would also like to thank Dr. Chang for expanding my knowledge in material science and applying my research to real life applications. I would like to thank Dr. Bonner for his excellence in scientific storytelling and for allowing me to continue to explore the biological realm.

I also thank all of the friends that I have made during my time here; you all have made Raleigh such a wonderful place to live and have contributed immensely to the establishment of my work-life balance. I would also like to thank my lab mates and collaborators for their support and assistance. Without you I would not have been able to finish this degree.

Finally, I thank my husband. He has always been a wonderful sounding board for questions about my research and kept me sane throughout this process. This has led to more than one evening of scribbling equations and chemical reactions on our bathroom mirror. He is smarter than he will ever give himself credit for, has an infinite well of knowledge to share, and is the most weird and wonderful partner I could have ever hoped to spend the rest of my life with.
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Figure 5.10. Illustration of proposed mechanisms underlying the acute phase immune response to ZnO-coated MWCNTs (Z-MWCNTs) in human THP-1 monocytes in vitro and after delivery to the lungs of mice in vivo by oropharyngeal aspiration. Uncoated MWCNTs (U-MWCNTs) are subjected to atomic layer deposition (ALD) coating with ZnO to yield Z-MWCNTs. Sonication results in breakage of Z-MWCNTs and the ZnO coating also undergoes partial dissolution to release Zn$^{2+}$ ions in aqueous media. Unlike U-MWCNTs, Z-MWCNTs are not taken up by phagocytic monocytes or macrophages in vitro or in vivo. Z-MWCNTs cause epithelial cell growth arrest and increases in CXCL10, a monocyte chemoattractant, as well as increases in IL-6 that mediates systemic acute phase responses.
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exhibited shivering, while mice exposed to MW were asymptomatic. Each treatment group (control, U-MWCNT, Z-MWCNT) contained 4, 5, and 5 animals, respectively.
CHAPTER 1. Introduction

1.1 Atomic layer deposition (ALD)

Atomic layer deposition (ALD) is the leading technique for the creation of thin films. With origins in Finland over four decades ago, ALD has withstood the test of time. The inventor of ALD, then called atomic layer epitaxy, was Tuomo Suntola; the first application was electroluminescent displays. Today ALD has many diverse applications including semiconductor fabrication; polymer coating, encapsulation and surface modification; carbon nanotube modification; nanopatterning; and organic electronics and printed photovoltaics.

ALD uses a set of self-limiting surface reactions to create thin, conformal coatings, see Figure 1.1. Precursors are added one at a time and allowed to react with the surface. Between each step a purge is used to remove all of the free chemical species. Cycles are repeated ABAB until the desired thickness is achieved. This process can be accomplished at a range of temperatures and pressures to achieve the desired coating.

Figure 1.1. Outline of atomic layer deposition (ALD), a set of two self-limiting half reactions leading to growth of one monolayer of material at a time. The reaction between diethylzinc (DEZ) and water are depicted here to deposit zinc oxide (ZnO).
1.2 ALD modifications

Over the past few decades ALD spin-offs have manifested themselves in academia. Moving on from simply coating a planar substrate, techniques are being created to make and use thin films in broader and more useful applications every day. The focus of this dissertation will be on techniques that go beyond traditional ALD.

![Diagram](image)

Figure 1.2. Outline of selective infiltration using trimethylaluminum (TMA) to pattern alumina. A) Infiltration starts with a patterned polymer on a silicon wafer. B) TMA gas is introduced to the system and held in the reactor to allow for diffusion and reaction to occur throughout the polymer bulk. C) The reactor is purged to remove unreacted TMA and byproducts. Steps 1-3 are repeated as many times as desired until D) one water dose, with no hold step, is introduced to form an oxide layer to trap in TMA. Samples were then removed from the reactor and E) heated rapidly in a furnace open to air to burn off the polymer, react the TMA with atmospheric water and anneal the product into a solid alumina layer.

The first technique is called selective infiltration, Figure 1.2. By changing the traditional ABAB ALD sequence to \([A \times n + B]\), infusion of the “A” precursor into the bulk of a polymer can be achieved and sealed in by a final “B” step. Infiltration can be encouraged further by adding a hold step where the precursor is held in the reactor to diffuse into and react with the
polymer. As outlined in Figure 1.2, this technique can be used on a nanopatterned polymer to create a nanocomposite of polymer and precursor.

Figure 1.3. Outline of ALD coating on MWCNT. 1) MWCNT are loaded into a mesh basket surrounded by nonwoven polypropylene (PP) and placed into an ALD reactor. 2) DEZ is dosed and held in the reactor and then 3) purged. 4) Water is then dosed into the reactor and held followed by 5) another purge. This can be repeated as many times as desired to coat the CNT with a thin film of ZnO, in the case described, or other materials.

The second technique challenges the viscous flow aspect of an ALD reactor. Coating high surface-area, nano-scale powders within an ALD reactor is close to impossible because the nanoparticles loaded into the reactor would be swept away with the first precursor dose. By encapsulating the powder within a mesh basket surrounded by an inert, permeable nonwoven like polypropylene (PP) the powder can be contained.6 Described in Figure 1.3, this technique involves the traditional ABAB ALD sequence but also features the hold mentioned in infiltration. This hold step allows the precursors to diffuse through the inert, permeable packaging to the powder where it can coat the surface of each nanoparticle within.
1.3 Nanopatterning via infiltration

The goal of this technique is to allow for dielectric patterning without dielectric etching. Patterning a polymer is faster and requires a less energy-intensive process than patterning a dielectric. In addition, sub-saturating infiltration can lead to pattern shrinking making it possible to pattern smaller features than are currently obtainable with standard patterning techniques. Our hypothesis states that by characterizing how the infiltration process parameters affect the resulting dielectric film we will be able to reliably pattern at the nanoscale across large surface areas.

Moore’s law predicted the doubling of the number of transistors in an integrated circuit roughly every two years. Although progress in transistor densification within integrated circuits has slowed recently, feature sizes are poised to reach and scale below 10 nm. This requires novel methods for patterning and aligning features. Several methods are currently under investigation including directed self-assembly, \textsuperscript{8–10} multiple e-beam direct write systems,\textsuperscript{11} and nanoimprint patterning.\textsuperscript{12–14}

Directed self-assembly uses the chemical thermodynamic driving forces inherent in block-copolymer layers to create molecular-scale patterns that can be transferred to an underlying active thin film. Compared to optical or physical lithography, such ‘chemical patterning’ methods can act over much smaller length scales and enable very precise feature alignment. Another option for chemical patterning is selective area deposition,\textsuperscript{15–22} where vapor- or liquid-phase precursors react only at desired locations to directly produce a patterned thin film. However, a basic understanding of selectivity, surface–chemical attraction and bonding is needed to achieve scalable and reliable manufacturing processes.

1.3.1 Selective deposition

To date the bulk of research into selective deposition has focused on chemical vapor deposition (CVD)\textsuperscript{21,22} despite ALD’s enhanced thickness control, film quality and conformality. In recent years more research has started to focus on selective ALD.\textsuperscript{15–20} Various methods for selective deposition using organic layers to block or promote film growth have been studied for selective
ALD of TiO$_2$, Ir, Pt, Ru, Al$_2$O$_3$, ZrO$_2$, HfO$_2$, AZO, ZnO, and PbS. For a thorough assessment of ALD patterning see the review by A.J.M. Mackus et al.

ALD is characterized by monolayer growth due to self-limiting surface reactions from brief gas exposures. Nucleation of this growth can be blocked or activated by polymers or self-assembled monolayers (SAM). The use of patterned polymers or self-assembled monolayers as blocking layers allows for the simultaneous deposition of features at various length scales. Unfortunately, ALD precursors are highly reactive and given enough time will often diffuse and react with the undesired, or blocked, surface leading to growth in unintended regions. After noting this behavior, infiltration has been capitalized upon to modify soft materials to enhance properties, create porous materials and form patterns.

1.3.2 Infiltration of ALD precursors

The intentional infiltration of ALD precursors, with or without co-reactants, into polymers is often accompanied by extended precursor exposures to allow for diffusion to occur. As this process no longer strives for self-limiting surface reactions to create thin films, it was given a new name. The first to publish a paper intentionally using this infiltration technique was Seung-Mo Lee et al. in 2009; it was called multiple pulsed vapor-phase infiltration (MPI) and was used to increase the toughness of spider silk. Soon after, in 2010, Qing Peng et al. selectively deposited ALD within the PMMA block of polystyrene-block-PMMA using extended ALD exposures which the group later dubbed sequential infiltration synthesis (SIS). Additionally, in 2011 Bo Gong et al. used this same technique, but called it sequential vapor infiltration (SVI), to create porous materials from infiltrating sacrificial polymers with ALD precursors. All three processes (MPI, SIS, and SVI) were characterized by long vapor exposures (either ABAB or just A) to promote diffusion into and modification of polymers. In this document these processes will be collectively referred to as infiltration as was recently done by Keith Gregorczyk et al.

As mentioned above, from this infiltration process another patterning process was born. Exposing a block co-polymer to extended TMA/water ALD cycles, for example, creates aluminum-oxygen sites bound within the reactive polymer region while the unreactive co-
polymer blocks nucleation. Selective infiltration of TiO$_2$, Al$_2$O$_3$, ZnO, SiO$_2$, and W have been accomplished using this method. Removing the organic by thermal oxidation or plasma exposure leaves behind nanoscale oxide lines that can function as a solid etch-resist. The oxide pattern is generally limited to parallel lines or pillars with limited thickness control. Furthermore, extending the number of process cycles beyond the optimum leads to metal oxide deposition within the less-reactive polymer layer, leading to film growth in undesired locations as was seen with the organic blocking layers with ALD.

1.3.3 Brief summary of results

Here we demonstrate that patterned polymers can be used to create nano- to micron-scale features simultaneously across large surface areas, similar to the organic blocking layers used to selectively inhibit ALD but instead using the organic as a positive template. To do this we too used extended vapor exposures to achieve saturated infiltration of the patterned polymer, like the block co-polymer process described above. As opposed to these other techniques, we were able to achieve selective patterning without a blocking layer and thus there was no critical limit to the number of cycles that can be used before selectivity is lost.

Our model system for this study was poly(methyl methacrylate) (PMMA), a common semiconductor photo resist, and trimethylaluminum (TMA), a standard ALD precursor for alumina.

First, a careful study of the interaction between TMA and PMMA was performed using in situ quartz crystal microbalance and infrared measurements along with quantum chemistry calculations. We found that at moderate temperatures, between 65 and 110 $^\circ$C, TMA and PMMA form a Lewis acid-base complex and at higher temperatures, between 110 and 150 $^\circ$C, undergo a six centered pericyclic reaction to form a covalent bond between PMMA’s pendant ester group and TMA.

We built upon the basic understanding of polymer/precursor interaction chemistry to use the polymer itself as the co-reactant. This allows us to adjust and control the extent of precursor reaction within the polymer and achieve full saturation, a step which is important to fully
reproduce features of different sizes on a single substrate. This selective, saturated infiltration process is shown schematically in Figure 1.2. We use an ALD reactor to expose a patterned polymer to multiple doses of a reactant (e.g. TMA) separated by an inert gas purge. Through extended exposures, the ALD reactant diffuses into and saturates the reactive sites within the polymer film bulk,\textsuperscript{38,39} thus “seeding” the conversion of the polymer to a metal oxide. After precursor saturation, a one second water exposure step ensures the infiltrated polymer is initially exposed to the same amount of water each time before being exposed to atmosphere for consistency. Following saturation the samples are annealed in air to remove the polymer and oxidize the TMA into alumina, leaving a metal oxide pattern where there was once a polymer pattern. Due to the self-limiting nature of the ALD reactant at the process temperature, substrate regions where the polymer coating has been removed are exposed to only one formal ALD cycle (with a long TMA dose step) limiting growth to the single-monolayer regime (i.e. a few Ångstroms thick).\textsuperscript{53}

We have created patterned films with thicknesses ranging from less than 10 nm to over 150 nm (limited by process time), and patterned features greater than 1 cm to as small as 20 nm (limited by available polymer patterning tools). We also show consistent and repeatable pattern generation across a 6 inch silicon wafer (limited by reactor size). We have also extended this technique to pattern titania by matching polymer reactivity with other vapor reactants.

Patterning of low k materials — which are used for its help in reducing resistance capacitance delay, power consumption, and cross talk of ultra large scaled integrated circuits — can be used to make interlayer dielectric insulators. Patterning of high k materials — which are used to increase gate capacitance, drive current and thus performance — can be used to make gate oxides that are becoming increasingly more geometrically complicated as manufacturers are forced to design transistors more creatively. Although we have targeted semiconductor applications for this technique, other applications include: templates for magnetic bit patterned media, heterogeneous catalysts, chemical sensing,\textsuperscript{54} polymer coating, encapsulation, and surface modification.\textsuperscript{2}
1.4 Modified nanomaterials and the lung

Using the second technique discussed above, we plan to test how ALD surface modification of multi-walled carbon nanotubes (MWCNT) with zinc oxide (ZnO) and aluminum doped zinc oxide (AZO) will affect the biological and toxicological responses of human lung cells (monocytes, fibroblasts, and epithelial cells) in vitro and mice in vivo.

The goal of this research was to determine how surface modification of MWCNT would affect the physical and chemical properties of the MWCNT and how these changes in physical properties would in turn affect the biological response to these particles. Surface modification of MWCNT is increasing as the push to find new and improved applications for MWCNT swells. A full body of knowledge must be created to allow for proper safe handling procedures of modified MWCNT in addition to their pristine counter parts. Our hypothesis stated that by fully characterizing MWCNT coating thickness, dissolution and composition as well as tube length, zeta potential and aggregate size we would be able to determine the relative effect of each parameter on the elicitation of a fibrogenic response. This will allow us to better predict health consequences of MWCNT with similar chemical and physical properties following surface modification.

This research is crucial for the safe design of engineered nanomaterials (ENM) due to the fact that MWCNT’s high aspect ratio and ability to readily disperse in the air is similar to that of asbestos. Asbestos is a well-known cause of pulmonary fibrosis, described in Figure 1.4, in humans. Numerous reports over a period of decades document lung tissue thickening and scarring (asbestosis) in asbestos miners, workers installing asbestos products (e.g., building insulation and brake linings), or in individuals living near asbestos mines. There is no cure for pulmonary fibrosis; prevention is the best and only way to keep people safe.\(^\text{3,55–58}\) No cases of MWCNT-induced pulmonary fibrosis have yet been documented but MWCNT have already been implicated in causing pulmonary fibrosis in rodent models.\(^\text{3,55,57–60}\) By studying the potential for MWCNT to cause similar problems, we can keep exposure to a minimum and prevent another epidemic of this life-threatening disease.
Figure 1.4. The secretion of extracellular matrix proteins by fibroblast cells is a necessary part of wound healing. Once the wound is healed, fibroblasts go through apoptosis to prevent overproduction of proteins. In pulmonary fibrosis, fibroblasts continue to survive and create proteins, leading to lung thickening. Thickening of the interstitial space of the alveoli leads to poor oxygen–CO₂ transfer between the lungs and blood stream.

1.4.1 Pulmonary fibrosis and inflammation

Pulmonary fibrosis is a chronic disease of the lungs caused by excess accumulation of extracellular matrix (ECM) proteins and altering of the lung architecture. The death of epithelial cells coupled with altered fibroblasts leads to imbalanced proliferation and apoptosis of fibroblasts as well as ECM creation and breakdown. Idiopathic pulmonary fibrosis is the most severe form with a mean survival of 3 years with no treatment options.

During the progression of pulmonary fibrosis, lung fibroblasts differentiate into myofibroblasts, the main secretors of the extracellular matrix protein collagen. Secretion of matrix proteins is a normal response to tissue injury that plays a role in wound healing. After wound healing, myofibroblasts then go through apoptosis, programmed cell death, to prevent the accumulation of unnecessary matrix proteins. In pulmonary fibrosis these cells survive and continue to secrete proteins.
Chronic inflammation is often associated with fibrosis. Inflammation is a response to injury, infection or toxic exposure. The goal of inflammation is to recover normal tissue function but unresolved it leads to tissue damage. Inflammation works to remove invaders from the body and restore normal function to the affected area. An increase in macrophages, neutrophils and eosinophils along with an increase in pro-inflammatory cytokines are often observed in inflammation.

Macrophages are one of the first lines of defense against foreign particles in the body and work to phagocytose invaders. When the foreign object is too large this can lead to frustrated phagocytosis or the fusion of multiple macrophages to form huge cells able to engulf the invader which in turn leads to the release of reactive oxygen species (ROS) and hydrolytic enzymes. ROS are often a component of inflammation. ROS lead to damage to DNA, proteins, cell membranes, and mitochondria. Oxidative stress can originate from environmental toxins, mitochondria or the depletion of antioxidants among other things.

Neutrophil influx is also characteristic of inflammation. Neutrophils are short lived granular leukocytes that also perform phagocytosis and can break down materials with enzymes, like myeloperoxidase, and have been known to degrade CNT. Eosinophils are less common white blood cells that are recruited to parasites, allergy and chronic inflammation; eosinophil peroxidase has been shown to degrade SWCNT.

### 1.4.2 Nanoparticle-lung interaction following inhalation

When nanoparticles (NP) are inhaled, the upper airway is cleared by the mucociliary escalator while the lower airway is cleared by macrophage phagocytosis of particles smaller than 10 μm followed by transportation to the mucociliary escalator or across the epithelium to the lymph. Neutrophils are also recruited to help clear nanomaterial. Macrophages are most able to clean materials that are close to their size (14-21 μm) with small (<100-200 nm) particles evading uptake. Following phagocytosis, phagosomes and lysosomes can combine to form phagolysosomes to break down the NP material. Particles that are not phagocytosed are more
likely to interact with the epithelial cell lining and lead to fibrosis. Increased residence times of materials leads to increased negative health effects.\textsuperscript{64,65}

Physical characteristics (size, shape, density) of NP can change where they deposit in the lungs with clearance and translocation dependent on geometry and surface area.\textsuperscript{64} Particles smaller than 10 \(\mu\)m can deposit in the alveoli while larger particles collect at larger branches. Increasing NP concentration can increase their aggregation and lead to increased clearance.\textsuperscript{65}

NP are likely to deposit in alveoli if smaller than 100 nm or between 100 nm and 1 \(\mu\)m and of high density. NP inhalation will exhibit impaired clearance if the NP are smaller than 100 nm, fiber shaped, surface charged, and/or have slow to no dissolution. Increased inflammation is observed if NP have a high surface reactivity, fast dissolution or are cationic.\textsuperscript{64}

\textbf{1.4.3 MWCNT uses and current research}

MWCNT are used in many different areas including: electronics, energy storage, sensors,\textsuperscript{3,60} organic light emitting diodes, transparent conductive coatings, capacitors, filtration,\textsuperscript{6} high strength materials, electronics,\textsuperscript{66} contrast imaging agent, drug delivery\textsuperscript{67} and flame retardants.

Lung exposure to CNT has led to fibrosis \textit{in vivo} using both mice and rats. Dong et al. exposed C57BL6 mice to 5-40 \(\mu\)g of MWCNT via pharyngeal aspiration and observed the initiation of fibrosis after only one day as characterized by increased collagen in the lungs. The severity of fibrosis peaked after seven days and was maintained to their latest time point of 14 days. They also observed acute inflammation with increased macrophages and neutrophils. MWCNT were primarily deposited in the terminal and respiratory bronchioles as well as the alveolar ducts.\textsuperscript{68}

CNT aggregate due to van der Waals forces. To disperse CNT they can be functionalized or ultra-sonicated. Ultra-sonication can break CNT into smaller pieces.\textsuperscript{69} Overcoming the van der Waals interactions between CNT is important for the creation of composite materials. CNT with short lengths have gained interest due to increased control over their contributions to strength and toughness in composites. Shortening increases dispersion because they become less entangled.\textsuperscript{70}
Long ridged MWCNT can lead to frustrated phagocytosis in macrophages which in turn leads to the release of ROS and hydrolytic enzymes.\textsuperscript{60} Some researchers believe that decreasing the length of a CNT will decrease toxicity because their smaller size would make them easier to clear. On the other hand, Mutlu et al. saw that long, dispersed CNT were clearable from the lung despite their aspect ratio. They hypothesize that aggregation of the CNTs causes toxicity and not the aspect ratio.\textsuperscript{71}

Muller et al. also focused on the effect of dispersion and length by using long and short MWCNT. MWCNT were shortened via grinding and then used to expose Sprague-Dawley rats intratracheally (0.5-5 mg) and found better lung dispersion with shorter tubes. Unground tubes were poorly dispersed, had increased bio-persistence and caused collagen-rich granulomas in the bronchi. Ground tubes were better dispersed and led to granulomas in the alveolar space or interstitium with slightly decreased bio-persistence and increased inflammation attributed to better dispersion.\textsuperscript{66}

In yet another study, the aggregate state was found to affect the biological response to CNT lung exposure. The extent and location of inflammation was dependent on the dispersal and aggregation of SWCNT with agglomerated tubes leading to granulomatous inflammation and fibrosis in the larger airways and dispersed SWCNT deposited in the alveoli and interstitium leading to peri-alveolar fibrosis.\textsuperscript{60}

Another study determined that dispersal state was an important characteristic in determining inflammation. Additionally, they found surface functionalization to be a key parameter as it can change the CNT length, aggregation and degradation. They also found that increased length and width of CNT increased inflammation.\textsuperscript{62}

\textbf{1.4.4 ZnO uses and current research}

ZnO is a wide band gap semiconductor\textsuperscript{72} with transparent, conductive, piezoelectric and UV absorbant\textsuperscript{73} properties. ZnO has a bandgap of 3.37 eV with a high excitation binding energy of 60 meV.\textsuperscript{74}
ZnO finds uses in cell imaging, bio-sensing, drug delivery, targeted cancer treatment, solar cells, sensors, transducers, photo catalyst, foods, pigments, cement, paint, cosmetics, sunscreen, antibacterial ointments, lotion, mouthwash, UV light emitting diodes, laser diodes, lasing media, optoelectronic devices in general, and piezoelectric generators when coated on CNT. It is also used as a dietary supplement because it stimulates the immune system and acts as an anti-inflammatory at the right concentration. As an antibacterial it is more effective against gram-positive bacteria than gram-negative.

In vivo zinc ions are coenzymes for many enzymes and are in zinc fingers found in transcription factors. Zinc is an essential trace element, one of the most abundant in cells. It is involved in cell structures, stabilization of cell membranes and catalytic functions. Without ZnO there is impaired immune function. But it is only good in moderation as excessive inhalation can lead to metal fume fever which has flu like symptoms.

As ZnO is photocatalytic and can be used to degrade toxic components of industrial effluents, ZnO is currently being studied as a filter supported on MWCNT. Taking advantage of ZnO’s piezoelectric properties, a MWCNT coated with ZnO could be used as a nanoscale battery charger or sensor when a mechanical stress is applied. For instance, a car moving across a bridge would cause the bridge to vibrate, these vibrations could be transformed into electricity. Coating MWCNT with ZnO is being studied as a nanogenerator. MWCNT coated with ZnO have been shown to make a stable, but reversible, super-hydrophobic material using a surface of aligned MWCNT. With such a broad range of applications there is a substantial potential for human contact and inhalation. MWCNT can affect the health of workers, consumers, and the environment.

ZnO is partially water soluble and the ions contribute to toxicity. For ZnO dissolution pH is a major factor. ZnO can react with water to form OH. Research has found that dissolution is time and concentration dependent; ZnO is not highly soluble without low pH or chelators. As a photocatalyst ZnO will make ROS with the aid of light with an energy at or above its band gap energy of 3.37 eV (corresponding to a wavelength of 368 nm).
Many people believe that the toxicity of ZnO is at least partially tied to Zn\(^{2+}\) release but the extent of which is unknown.\(^{73}\) Some people believe that ZnO toxicity is dependent on ROS formation, enzyme activity inhibition, and lysosomal/mitochondrial damage from altered Zn\(^{2+}\) homeostasis.\(^{73}\)

Cho et al. studied the contribution of Zn\(^{2+}\) by exposing rats intratracheally to ZnO NP at 50 and 150 cm\(^2\)/rat or Zn\(^{2+}\) ions at 92.5 and 277.5 \(\mu\)g/rat and looked at the response after 24 hours, one week and four weeks. They found the responses to be similar between the two with increases in eosinophilia, proliferation of epithelial cells, goblet cell hyperplasia and pulmonary fibrosis. They believe the similarity stems from the fact that the ZnO NP are being engulfed by macrophages and dissolved in lysosomes, thus allowing the NP to quickly change to ions. One difference they did observe was that the high dose of Zn\(^{2+}\) led to rat death were the high dose of ZnO NP did not.\(^{75}\)

Many others have concluded that Zn\(^{2+}\) plays a role but saw differences between ions and NP.\(^{75,82}\) For example, another group exposed A549 lung epithelial cells \textit{in vitro} as well as rats \textit{in vivo} to ZnO NP and Zn\(^{2+}\) to look at the contribution of Zn ions to inflammation. To create the ionic conditions, media was incubated with ZnO NP and then filtered to remove the NP. \textit{In vitro} they found that both were cytotoxic but the ions alone were less cytotoxic and produced a smaller increase in IL-8. In rats, exposure to ZnO NP increased bronchoalveolar lavage fluid (BALF) levels of neutrophils and eosinophils; LDH and total protein after 24 hours were observed; and levels of increased total cells and eosinophils persisted to 28 days. Ions alone showed an increase in neutrophils at 24 hours with everything back to baseline by 48 hours.\(^{83}\) This led them to believe that the response seen was not from ions alone.

Moos et al. determined that ZnO NP contact was necessary to induce cytotoxicity in human colon RKO cells. Using 40 and 330 nm ZnO NP and comparing the dosing of cells directly, dosing on the other side of a cassette with no direct cell contact and dosing using a trans-well membrane where ZnO NP were in the lower membrane they were able to show that ion levels were not sufficient to induce cell death or interrupt mitochondrial function without ZnO NP contact. The Zn\(^{2+}\) concentration was similar between trans-well and direct case and between
media alone (without cells) and cassette case. Moos et al. cited cellular factors as likely assisting in the dissolution of ZnO and these factors could not fit through the 10kDa cassette. The also observed higher apoptosis and decreased mitochondrial function for the smaller particles dosed on a mass basis.\textsuperscript{81}

Buerki-Thurnherr et al. exposed Jurkat cells to ZnO NP and believe that cytotoxicity was not caused by ROS but from ZnO dissolution in the media. They found that N-acetyl cysteine (NAC) was the only antioxidant of four tested found to decrease cytotoxicity. NAC is a known chelator of Zn\textsuperscript{2+} so it was likely acting by sequestering ions and not as an antioxidant, thus pointing to ions and not ROS being the key factor in cytotoxicity. To look into dissolution, ZnO NP were coated to reduce the dissolution rate and this led to decreased cell death. The authors decided that dissolution occurred in the media as they could not visualize any NP in the cells via transmission electron microscopy. They concluded that ZnO NP toxicity was from ZnO dissolution extracellularly followed by cellular uptake of ions and ROS independent apoptosis.\textsuperscript{84}

Xia et al. looked into the contribution of ROS and Zn\textsuperscript{2+} to the toxicity of ZnO NP to RAW 264.7 macrophages and BEAS-2B epithelial cells. ZnO NP led to increased toxicity, ROS, oxidant injury, inflammation, and cell death in both cell lines. ZnO was found in caveolae in epithelial cells and in lysosomes in macrophage cells. They found that Zn\textsuperscript{2+} from ZnSO\textsubscript{4} were more toxic than ZnO NP highlighting that there is a contribution of more than just ions from the NP.\textsuperscript{85} The same group later continued to study ZnO NP with epithelial BEAS-2B cells and concluded that ZnO NP were engulfed by the cells where they dissolve and create ROS followed by cell death. They also determined that Zn\textsuperscript{2+} could complex with organic molecules, especially thiols. In this second paper they doped ZnO NP with iron to slow dissolution and used AFM to determine that ZnO NP were both associated with the outside of cells and found within cells. This was not possible with un-doped ZnO NP because of their quick dissolution within cells.\textsuperscript{86}
AZO uses and current research

Alumina (Al) doping increases the conductivity of ZnO by making electrons more easily excited into the conduction band with the highest conductivity resulting from 3-7% Al. Alumina doped ZnO (AZO) is the best candidate to replace indium tin oxide (ITO) as the premier transparent conductive oxide (TCO). TCOs are necessary for transparent devices such as solar cells, flat panel displays and light emitting diodes. Currently ITO is the most commonly used TCO because it has a low resistivity and high transmittance, but indium is rare and toxic and ITO is generally unstable. AZO is a promising substitute with high transmittance and low resistivity with the added bonus that Al is abundant and nontoxic. AZO is more stable, cheap and environmentally friendly replacement for ITO.

AZO has decreased solubility and crystallinity as compared to pure ZnO. After 24 hours of incubation in cell media the concentration of Zn$^{2+}$ in cell media for AZO NP was half that of ZnO NP in one study with others also observing a decrease in Zn$^{2+}$ in solution with Al doping. Al doping of ZnO alters the crystallinity of ZnO; with a high enough concentration of alumina films become amorphous and with just a small amount films exhibit decreased surface roughness and crystallinity.

Less research has been done to explore the toxicity of AZO NP. The few studies that have been done contradict one another with some claiming AZO NP to be less toxic than ZnO NP and vice versa.

Xu et al. tested a group of NP made up of insulators and semiconductors including ZnO and AZO (3.4% Al) NP. They found that NP treated media had no effect on NIH3T3 mouse fibroblast cells or A549 human lung epithelial cells. This ruled out ions as major contributors to the cellular response and thus changes in solubility upon doping were not considered important. They found that the AZO NP were more toxic than the ZnO NP.

Akhtar et al. discovered that MCF-7 breast cancer cells were more sensitive to AZO NP (3% Al) created using a sol gel method than to ZnO NP. Cells showed increased cytotoxicity, loss of mitochondrial membrane potential and increased oxidative stress. The authors claim ROS
to be a contributing factor to the cellular response because the antioxidant NAC increased cell viability. Unfortunately, NAC’s ability to chelate Zn$^{2+}$ means the contribution of ions verses ROS remains poorly understood. They hypothesize that the creation of ROS through a mitochondrial pathway is the main mechanism of MCF-7 toxicity. They went on to find that IMR-90 human lung fibroblasts and primary rat hepatocytes were not affected by treatment with either NP.

Pan et al. showed that AZO (purchased, 2% Al) was less cytotoxic than ZnO NP and saw a good correlation between zeta potential and viability. This contradicts the first study showing that AZO was more toxic than ZnO.

1.4.6 Brief summary of results

Previously Taylor et al. found that coating MWCNT with alumina via ALD decreased the fibrotic response in mice exposed via oropharyngeal aspiration. We sought to determine if other ALD coatings would also change the fibrotic response in mice in vivo or in cell culture in vitro.

The first coating tested was ZnO. In vitro we studied the cellular response using human monocytes (THP-1 cells). THP-1 cells are often used to model the innate immune system and the human inflammatory response. It was found previously that particle number is the best dosing strategy for nanomaterials. As such, cells were dosed by normalizing to the nanoparticle number as the coating significantly increases the weight of the MWCNT.

THP-1 cells were exposed to MWCNT, ZnO coated MWCNT (Z-MWCNT) and ZnO NP. After 24 hours Z-MWCNT- as well as ZnO-NP-exposed cells exhibited increased levels of mRNA for the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, with significant increases of the two former cytokines. Significant increases of CXCL10 were also observed. No increases were observed for cells treated with MWCNT. These results indicate an inflammatory response to both nanomaterials containing ZnO.
This inflammatory response *in vitro* matched our results *in vivo*. After exposing C57BL6 mice to MWCNT and Z-MWCNT via oropharyngeal aspiration, an acute inflammatory response was observed in mice exposed to Z-MWCNT and to a lesser extent MWCNT. There was an increase in total cells in the BALF and neutrophils observed from both nanomaterials with a significant increase in macrophages and neutrophils from Z-MWCNT. This was coupled with a significant increase in IL-6 and CXCL10 mRNA and protein in Z-MWCNT exposed mice. Systemic increases in IL-6 were also observed in Z-MWCNT treated mice with significant increases in the heart and liver.

Although the initial inflammatory response was more severe with Z-MWCNT treatment, by day 28 both showed similar cell profiles in the BALF and baseline cytokine levels. Levels of fibrosis were similar between the two treatments with the main difference being the location of fibrosis. Treatment with MWCNT caused more focal, condensed lesions at alveolar duct bifurcations whereas Z-MWCNT caused a more diffuse inflammatory response in the lower lung around terminal bronchioles, alveolar duct bifurcations and alveolar ducts. This initial inflammatory response is likely due to the presence of ZnO, which has been known to cause inflammation, and the change in physical properties of the MWCNT upon coating. Coated tubes are smaller, better dispersed and denser. All of these factors could lead to better dispersion in distal regions of the lung. The longer, more aggregated MWCNT deposit higher in the airway.

To mitigate the aggressive inflammatory response to Z-MWCNT we sought to alter the ALD coating. Mixed results had been seen previously with alumina doping of ZnO to create AZO NP. As we previously observed ALD alumina coatings to decrease the level of fibrosis as compared to uncoated MWCNT, we thought that doping would be an interesting place to start.

We used AZO coatings to test the contribution of Zn$^{2+}$ to toxicity as AZO is less soluble than ZnO. To study the ion release we incubated A-, Z- and AZO-MWCNT in media alone or with various cell lines. Zn$^{2+}$ concentration was partially dependent on the cell line, pH of the media and cellular uptake of the nanomaterial. Increased ion concentrations led to increased cytotoxicity. Cytotoxicity from AZO-MWCNT treatment was less than or equal to that of Z-
MWCNT treatment. When differences in Zn$^{2+}$ concentration were observed between Z- and AZO-MWCNT treatments there were also differences in the release of the pro-inflammatory cytokine IL-1β. Together, these results mean that Zn$^{2+}$ contributes to toxicity and inflammation but the concentration is dependent on biological factors.
CHAPTER 2. Temperature-Dependent Reaction Between Trimethylaluminum and Poly(methyl methacrylate) during Sequential Vapor Infiltration: Experimental and Ab Initio Analysis


2.1 Introduction

Several active research groups currently explore polymer modification by vapor-phase, metal-organic reagents to understand reactions that alter material surface and bulk structure as well as functionality. Improved understanding of vapor infusion and reaction mechanisms will help expand the use of current methods, and will lead to the discovery of novel approaches or process schemes to enable new and broader applications. For example, sequential vapor infiltration (SVI) proceeds by repeatedly exposing a polymer, or other material, to a vapor reactant, usually a metal-organic species, in a heated reactor environment. After the reagent vapor flows into the reactor the deposition chamber is closed for a set “hold” time, increasing the net reactant exposure. Sequential exposures are separated by an inert gas purge to remove vapor byproducts and renew the reactant concentration. Co-reactants, such as water to produce metal oxide products, are also delivered either as an additional step within the reactant/inert gas sequence, or after completing the desired number of reactant infusion/purge cycles. This approach has grown from atomic layer deposition (ALD), which uses sequential, self-limiting reactant exposure steps to deposit conformal and uniform thin films on surfaces with monolayer precision. Variations on the process take different names, including multiple pulse infiltration and sequential infiltration synthesis. Multiple pulse infiltration also uses a hold step after precursor exposure, whereas sequential infiltration synthesis typically uses co-reactant exposures after each precursor step, without hold steps.
Infiltration and reaction involving trimethylaluminum (TMA) vapor and the polymer poly(methyl methacrylate) (PMMA), the latter in a thin film or block co-polymer (BCPs) such as polystyrene-block-poly(methyl methacrylate) (PS-b-PMMA), is a commonly studied system. PMMA has reactive functional groups and a relatively large free volume. TMA is a common reagent for ALD of Al₂O₃; its small size and high reactivity make it a good candidate to study vapor infusion and reaction processes. Several recent studies show that TMA exposure to PS-b-PMMA BCPs lead to selective reaction within the PMMA which, upon subsequent plasma oxidation, produces a solid template of the original PMMA that can be used for pattern transfer and lithography.

Even with this interest in vapor infusion, the detailed chemical mechanisms associated with the reaction remain uncertain. A recent study from our group using in situ infrared transmission (IR) showed TMA infusion and reaction depends strongly on the nature of the starting polymer structure. Moreover, based on changes in IR, we hypothesized mechanisms for TMA/polymer reactions that included covalent bond formation between TMA and PMMA at moderate process temperature (80°C).

In this article we present more detailed results including temperature dependent IR, quartz crystal microbalance (QCM) mass uptake, and quantum chemical modeling analysis. We also show that at moderate temperatures TMA forms a non-covalent, metastable adduct coordinating to the PMMA carbonyl unit. TMA either desorbs from this coordinated complex under purge conditions or subsequently reacts, more readily at higher temperatures, to form a covalent Al-O bonding structure that remains present after water exposure. New insight from this analysis will be helpful to understand reaction products and mechanisms for this and other vapor-phase metal-organic interactions with polymers.

2.2 Experimental procedures

2.2.1 Chemicals and materials

Poly(methyl methacrylate) (Fluka Analytical, MW 97,000) and trimethylaluminum (Strem Chemicals, min 98% pure) were used as received. TMA was co-reacted with deionized water
after a specified number of TMA doses. The reactor was purged with high purity nitrogen gas (Machine & Welding Supply Co) that was further purified with an Entegris GateKeeper located directly upstream from the reactor input. PMMA was spun-cast onto silicon substrates (University Wafers, P-type, <100>) by first dissolving PMMA into toluene (Fischer Scientific) from one to eight weight percent PMMA. Silicon wafers were cleaned with acetone (Fischer Scientific) and dried by spinning at 2000 rpm for one minute. The surface of the silicon was flooded with PMMA solution and then spun at 2000 rpm for one minute. The sample was then heated on a hot plate at 200°C for two minutes to remove toluene.

2.2.2 Sequential vapor infiltration (SVI)

SVI was used to infiltrate the PMMA thin film. Briefly, the sample was placed into a custom made, viscous-flow, hot-walled, vacuum reactor described previously\textsuperscript{99,100,108} The reactor was kept at roughly 800 mTorr, and operated at temperatures between 45 and 150 °C. TMA was introduced into the reactor and held by closing all ports into and out of the reactor for a set time period. The reactor was then purged with N\textsubscript{2} gas. This was repeated \( n \) times followed by a dose of water and a final purge. We anticipated that the final water step would help form a protective barrier on the outside of the polymer-TMA composite to block the rapid reaction of trapped TMA with atmospheric water. The typical dosing scheme for these experiments was a 1 second TMA dose, 60 second hold, and 30 second purge, all repeated as many times as desired. This is followed by a 1 second dose of water and a 45 second final purge. This scheme is denoted as \([(1/60/30) \times n + (1/45)]\). For the duration of this article a “TMA dose” will refer to one TMA dose, hold and purge step and will be reported as a number of repeats of the TMA dose (\( n \)).

2.2.3 Characterization

PMMA layer thickness was determined by spectroscopic ellipsometry (J.A. Woollam Co., Inc) and by profilometry (Veeco Dektak 150). Mass gain was quantified using an \textit{in situ} quartz crystal microbalance. For QCM analysis, 50 nm PMMA films were cast onto quartz crystals (gold plated, 6 MHz resonant frequency, Inficon). They were then affixed to a crystal drawer
(Inficon) with conductive silver epoxy (MG Chemicals). The crystal drawer was then inserted into the sensor head and placed into the reactor. The set up was allowed to equilibrate at process temperature overnight. The oscillating frequency of the crystal was recorded every 150 milliseconds and converted into a mass gain using the Sauerbrey equation.

Changes in chemical bonding were measured using an in situ Fourier transform infrared spectrophotometer (Nicolet 6700 FTIR) incorporated into a custom bu. Samples were cast onto IR transparent silicon wafers. The sample was prebaked in the reactor to a temperature of 140°C for 30 minutes under vacuum and flowing N₂ gas to remove any volatile species from the PMMA. The system was then cooled to the infiltration temperature and allowed to equilibrate for 30 minutes. Spectra were taken after 10, 50, 100, and 150 TMA doses as well as after water dosing. After the prescribed number of TMA doses the chamber was purged for two minutes and then closed off. Gates to the IR windows were then opened and 200 spectra were obtained at a resolution of 4 cm⁻¹ in the frequency range of 4000 to 400 cm⁻¹. An MCT-A detector was used through CsI IR windows.

2.2.4 Quantum chemistry analysis

Optimized geometries and frequencies of PMMA, TMA, and the PMMA–TMA coordination complex were calculated using the B3LYP Density Functional Theory method implemented because of its ability to accurately predict frequency data through the use of a scaling factor. All calculations were performed using a 6-31G++(d,p) basis set. This basis set provides suitable accuracy and rapid system output. As is commonly done with the B3LYP we used a multiplication factor of 0.961 to adjust the calculated frequencies to better replicate the experimental values. The as-calculated and adjusted values are reported to construct the model structures and view the calculated vibrations to identify modes in the experimental spectra. All simulations used methyl trimethylacetate as a model molecule for PMMA due to its structural similarity to one PMMA repeat unit. It has been shown that methyl trimethylacetate has a similar carbon
2.3 Results

2.3.1 In situ FTIR and QCM

Figure 2.1 shows in situ FTIR results following 150 TMA doses on PMMA films between 45 and 150°C. Each spectrum was collected from a separate experiment, starting each time with a fresh, spun-cast PMMA film. Spectra are shown in differential mode relative to the starting PMMA substrate. The starting PMMA spectrum shows peaks at 1732, 1260 and 1143 cm\(^{-1}\) (noted with dashed lines in Figure 2.1). The spectra show notable changes upon TMA exposure, especially in the region between 1100 and 1800 cm\(^{-1}\). The magnitude of mode intensity change upon TMA exposure could depend on temperature, TMA partial pressure, exposure time, purge time, and starting layer thickness. Therefore, to address the effect of temperature, we fixed the pressure, exposure, purge time and substrate thickness (< ± 1%) for all spectra collected. With these considerations, the distinct mode changes in Figure 2.1 reasonably relate to different extents of reaction, or different reaction products produced at the temperatures studied.

Previously, we reported in situ IR analysis of reactions between TMA and various polymer thin films, including PMMA, at a fixed temperature (80°C);\(^{107}\) spectra were similar to those at 70 and 90°C shown here in Figure 2.1. We note here that PMMA modes can be seen for C=O stretching at 1732 cm\(^{-1}\), and for C-O stretching in the =C-O- and -O-CH\(_3\) units near 1260 and 1143 cm\(^{-1}\) (vide infra). Consider first the effect of TMA exposure at 70°C on the IR results in Figure 2.1. After 150 TMA exposure steps, the differential spectrum shows negative going modes at 1732, 1260 and 1143 cm\(^{-1}\), and positive going modes at 1670, 1305, and 1200 cm\(^{-1}\), indicating significant modification of the C=O and C-O bonds. We previously observed and quantified changes in IR signature upon TMA exposure, concluding that TMA readily diffuses and reacts in the polymer bulk. That work also reported changes in the C=O and C-O modes\(^{107}\) where we assigned the mode near 1600 cm\(^{-1}\) (at 1670 cm\(^{-1}\) in Figure 2.1) to the reaction between TMA and the C=O forming O-C-O-Al units. Based on experimental and modeling results discussed in detail below, we revise this interpretation and assign the mode at 1670 cm\(^{-1}\) to TMA complexed to the ester C=O in a Lewis acid-base adduct\(^{115}\) Physisorbed TMA will...
also indirectly affect the non-carbonyl oxygen of the ester moiety via resonance, resulting in shifts in the C-O modes.

Figure 2.1. *In situ* FTIR spectra collected after 150 TMA doses on 170 nm thick PMMA films on silicon. The spectra are shown in differential mode, relative to the starting PMMA. Two spectra collected after TMA + water at 70 and 150 °C are also included, and similarly referenced to the starting PMMA.

Considering the data in Figure 2.1 collected at T= 45°C, there is much less change in the IR modes than at 70°C. For T >70°C the 1670 cm⁻¹ mode also appears but it is less intense at increased temperature. Moreover, at T = 110°C a small mode becomes visible at 1568 cm⁻¹ and it intensifies as the reaction temperature increases to 150°C. The identity of the 1568 cm⁻¹ mode is discussed in detail below.
In addition to spectra collected after TMA exposure, Figure 2.1 shows IR traces collected after water exposure (referenced to the starting PMMA) following TMA at 70 and 150°C. At 70°C, the TMA/water exposure sequence produced only small net changes in the PMMA features, indicating that the water step reverses or removes much of the change that occurred during the TMA step. TMA in the film will react with water to create Al-O clusters, consistent with the small broad feature near 820 cm\(^{-1}\) due to Al-O stretching. The TMA/water sequence at 150 °C produces a very different outcome. The TMA step produces a negative-going peak at 1732 cm\(^{-1}\) and a positive-going mode at 1568 cm\(^{-1}\), and it remains present after the water step (i.e. the spectra labeled “TMA” and “Water” at 150 °C look relatively similar). These results suggest the TMA forms a reversible product with the PMMA at low temperature (removed by water exposure), but at high temperature TMA reacts with the polymer to form a stable covalent product (not strongly modified by water). The =C-O- and -O-CH\(_3\) modes at 1260 and 1143 cm\(^{-1}\) in the starting PMMA show similar trends. At 70°C, the TMA exposure leads to changes that are largely reversed by the water dose, whereas at high temperature, the TMA leads to changes that are relatively stable upon water exposure (i.e. the spectra labeled “TMA” and “Water” are very different at low temperature, but are more similar at 150°C). In the Discussion section below, we consider possible PMMA–TMA reaction mechanisms and covalent bond products that can account for these IR results.

To further explore the reaction between TMA and PMMA, we performed quartz crystal microbalance (QCM) measurements during repeated TMA exposures to 50 nm spun-cast PMMA films at various reaction temperatures; results are shown in Figure 2.2. The QCM response over 60 TMA doses (Fig. 6.2a) show rapid net mass uptake during the first ~10 doses followed by slower uptake. For the three temperatures shown the net mass uptake is largest at 100 °C. Each TMA dose produced mass uptake followed by mass loss. The traces collected during TMA doses 41-43 (Fig. 6.2b) show that after the initial mass uptake the mass loss is nearly equal to the mass gain, with the largest gain and loss at 100°C. The trace shape also varies with temperature. At 140°C the mass gain jumps rapidly then continues more slowly. At 100°C the mass increases continuously, whereas at 70°C the mass uptake shows a peak followed by a small loss before continuing to grow. The QCM traces show TMA uptake and
loss from the PMMA bulk during each hold/purge step. The trace shape at 70°C is ascribed to relatively small amounts of TMA rapidly adsorbing and desorbing on the film surface, combined with slower TMA diffusion in and out of the PMMA bulk. Also, using QCM we find that after 50 dose steps at 70°C a long (85 min) purge led to a loss of 60% of the mass gained, whereas the same experiment at 150°C led to a mass loss of around 30%. At 150°C the mass loss plateaued after purging for approximately 15 minutes; at 70°C the mass loss did not plateau during the extended purge. This mass uptake/loss during TMA exposure on polymers, including PMMA, has been observed but has not previously been analyzed as a function of process temperature.

![Figure 2.2](image-url)  
Figure 2.2. (a) Overall QCM mass response for 50 nm PMMA films exposed to 100 TMA doses at 70, 100 and 140 °C. (b) Magnified view of the mass response after 41-43 TMA doses.

To further understand the trend in mass uptake versus exposure and temperature, we measured in situ IR absorbance versus TMA exposure at 70 and 150°C, as shown in Figure 2.3. The figure shows differential spectra relative to the previously plotted spectrum, where negative-
going features correspond to modes removed and positive-going features to modes added. The spectrum labeled “10 doses TMA” corresponds to changes that occur between 0 and 10 doses, whereas “50 doses TMA” shows changes between 10 and 50 doses. Considering the data collected at 70°C we see large changes during the first 10 doses including: loss of C=O stretching at 1732 cm\(^{-1}\); loss of C-O at 1260 and 1143 cm\(^{-1}\); and gains at 1670, 1305, and 1200 cm\(^{-1}\). This change continues, but is less pronounced for the next 40 doses with only small changes appearing after 100 and 150 TMA doses. At 150°C, these same changes are observed with the addition of a new mode appearing at 1568 cm\(^{-1}\) after 10 doses (also seen in Figure 2.1) which was not observed at 70°C. After 40 more TMA doses at 150°C the spectrum shows a further decrease at 1732 cm\(^{-1}\). The mode at 1670 cm\(^{-1}\) is now negative-going with an increase at 1568 cm\(^{-1}\). Note that the feature at 1670 cm\(^{-1}\) that disappeared after 50 TMA doses at 150°C is the same mode that appeared during the first 10 doses at both 150 and 70°C. The degradation of the 1670 cm\(^{-1}\) mode during TMA exposure was only observed at elevated temperature in our experiments. This trend—peak appearance followed by its disappearance—indicates the formation of a metastable intermediate (mode at 1670 cm\(^{-1}\)) that reacts at elevated temperature to create a more stable product state with a C=O mode at 1568 cm\(^{-1}\). Between 50 and 150 TMA doses we continue to observe the loss of the PMMA C=O mode at 1732 cm\(^{-1}\) and corresponding gain in the 1568 cm\(^{-1}\) feature consistent with stable product formation at the higher temperature.
2.3.2 Quantum chemical analysis

We performed quantum chemistry calculations as described above to minimize the potential energy for a PMMA analog, TMA and various possible reaction-product states. Figure 2.4 shows chemical structures for the species modeled, and the corresponding vibrational spectra. The calculated spectrum for the PMMA model (Fig. 6.4a) shows peaks at 1790, 1307, 1212 and 1182 cm\(^{-1}\). The peak at 1790 cm\(^{-1}\) results from the C=O stretch, and the other three peaks correspond to coupled =C-O- and -O-CH\(_3\) stretching modes. Carbon-hydrogen stretching and deformation modes are also present at 3000-3200 and 1400-1550 cm\(^{-1}\), respectively.
starting PMMA spectrum in Figure 2.1 shows peaks at 1732 cm$^{-1}$ (C=O stretch), and at 1260 and 1143 cm$^{-1}$ (=C-O- and -O-CH3 stretch). Table 2.1 lists the experimentally observed and calculated peak positions (as output and after frequency adjustment) for PMMA before and after TMA exposure. For the starting PMMA, the calculated peaks match well with the experimental values. An additional peak predicted near 1165 cm$^{-1}$ is not distinctly observed, but it could correspond to a shoulder that appears near 1189 cm$^{-1}$ on the 1143 cm$^{-1}$ peak.
Figure 2.4. Relaxed chemical structures calculated using \textit{ab initio} modeling, and their corresponding vibrational spectra referenced to spectra from structures calculated without TMA interaction. The bond models used for the calculations are also shown. The calculated spectrum for the PMMA model shows peaks at 1790 cm\textsuperscript{-1} corresponding to C=O stretch, and features at 1307, 1212 and 1182 cm\textsuperscript{-1} associated with =C-O and -O-CH\textsubscript{3} coupled modes. For each structure, interaction with TMA leads to loss of the 1790 cm\textsuperscript{-1} C=O mode and changes in the C-O vibrations. The peak at 1725 cm\textsuperscript{-1} is TMA coordinated to the C=O in a physisorbed state, and is consistent with the IR and QCM results at lower temperature. Peaks between 1750 and 1500 cm\textsuperscript{-1} in products B and C correspond to C=O coordinated with a neighboring covalently-bound Al-O, forming a resonant C=O\cdots Al-O-C unit, consistent with the higher temperature product mode observed at 1568 cm\textsuperscript{-1}. 
Table 2.1. Experimental, calculated and adjusted IR peak positions for methyl trimethylacetate as a model for PMMA. Experimental and calculated peak positions are also shown for the starting material after exposure to trimethylaluminum.

<table>
<thead>
<tr>
<th>Material</th>
<th>Experiment</th>
<th>Calculated</th>
<th>Adjusted</th>
<th>Vibration</th>
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</thead>
<tbody>
<tr>
<td>PMMA</td>
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<td>1790 cm(^{-1})</td>
<td>1720 cm(^{-1})</td>
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<td></td>
<td>1260</td>
<td>1307</td>
<td>1256</td>
<td>=C-O- and -O-CH(_3)</td>
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<td></td>
<td>--</td>
<td>1212</td>
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<td></td>
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<td></td>
<td>1143</td>
<td>1182</td>
<td>1136</td>
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<tr>
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<td>1725</td>
<td>1658</td>
<td>C=O-{Al(CH(_3)(_3)}</td>
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<tr>
<td></td>
<td>1305</td>
<td>1323</td>
<td>1271</td>
<td>=C-O- and O-CH(_3) with C=O-{Al(CH(_3)(_3)}</td>
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<td>1568</td>
<td>1605(^b)</td>
<td>1542(^b)</td>
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</tbody>
</table>

\(^a\)The scaling factor used was 0.961. \(^b\)Calculated and adjusted values are from structure \(C\) in Figure 2.4.

Previous quantum chemistry analysis of TMA interacting with surface hydroxyls during TMA/water ALD shows that TMA forms a short-lived metastable -O···Al(CH\(_3\)\(_3\)) adduct that subsequently reacts exothermically to yield stable -O-Al(CH\(_3\)\(_3\)) and methane\(^{117}\). Also, since TMA is a strong Lewis acid\(^{118}\), we minimized the energy for TMA physisorbed to the C=O through a Lewis acid-base adduct state, producing the structure and corresponding vibrational spectrum shown in Figure 2.4. Like the experimental results, the calculated spectrum was plotted relative to the starting PMMA. The calculated spectrum for PMMA+TMA shows negative-going modes at 1790, 1307, 1212 and 1182 cm\(^{-1}\) and new positive-going features at 1725, 1323, 1237 and 1197 cm\(^{-1}\). A peak near 730 cm\(^{-1}\) corresponds to Al-methyl rocking modes in the Al-CH\(_3\) groups. The Gaussview model confirms that the 1725 cm\(^{-1}\) peak corresponds to stretching of the C=O unit coordinated with Al(CH\(_3\)\(_3\)). Likewise the 1323, 1237 and 1197 cm\(^{-1}\) peaks correspond to =C-O- and -O-CH\(_3\) coupled vibrations neighboring the -C=O···Al(CH\(_3\)\(_3\)) coordinated structure. A shift in the C=O frequency to smaller wavenumber is expected with an increase in the C=O bond length, which is consistent with TMA coordination. The coordination will also change the =C-O- and -O-CH3 bond lengths, shifting the mode frequencies. These modes are expected to appear in the experimental spectra near 1658, 1271, 1189, and 1150 cm\(^{-1}\), respectively, as indicated in Table 2.1.
The experimental data collected at 70°C shows three clear positive-going modes at 1670, 1305, and 1200 cm\(^{-1}\) after TMA exposure. As in the PMMA spectrum before TMA exposure, the calculated peak near 1150 cm\(^{-1}\) is not readily observed. The predicted peaks after TMA exposure are at generally smaller wavenumber than experimentally observed, *i.e.* the errors between the adjusted and measured peak positions are somewhat larger after TMA exposure than for neat PMMA. Since the calculations do not include thermal or matrix effects, and the base cluster size utilized was relatively small, we expect the calculation to exaggerate the interaction, shifting the peaks more than experimentally measured. With this consideration, the adjusted peaks agree well with the experimental values. The other frequencies included in Table 2.1 are discussed below.

### 2.3.3 Observed chromatic shift

Bulk PMMA powder was exposed to TMA vapor at 90 and 150°C in a fiber-encapsulated basket placed inside our reaction chamber, as previously described.\(^{119}\) PMMA powder as received is white, as shown in Figure 2.5a. Upon 600 TMA doses at 90°C, no color change was observed. When the reactor temperature was increased to 150°C, the powder appeared black upon removal from the reactor. Faint popping sounds could be heard as the black powder converted to orange, consistent with a rapid reaction between the TMA–PMMA composite and water in the air. After 24 hours the powder was reddish brown. The sample maintained a stable orange color for more than 9 months following exposure, as shown in Figure 2.5f. Color changes due to metal atoms bonded and coordinated to organic groups are often used as metal-complex dyes.\(^{120}\) Complex-dyes have structures similar to that of alumina bound to multiple PMMA pendent groups as depicted in Figure 2.4c.
Figure 2.5. Bulk PMMA powder, as received, was treated with 600 TMA doses at varying temperatures. At increased temperatures a color change was observed that was not seen at lower temperatures. A) PMMA powder as received. B) PMMA powder with 600 SVI TMA doses at 90°C. C) PMMA powder with 600 SVI TMA doses at 150°C immediately after removal from the reactor, after D) 1.5 hrs E) 24 hrs and F) 9 months.

2.4 Discussion

Based on the IR and QCM data combined with the ab initio analysis results, we conclude that over a wide temperature range TMA diffuses into the polymer bulk and forms a metastable adduct, coordinating to the PMMA ester carbonyl. The physisorbed TMA shifts the C=O stretching frequency from 1732 to 1670 cm$^{-1}$, which appears in the difference spectra in Figure 2.1 as a mode loss adjacent to a mode gain. By interaction with the carbonyl oxygen, the TMA Lewis acid increases the C=O bond length and decreases the stretching frequency. The change in the C=O unit also draws some charge from the =C-O- and -O-CH$_3$ bonds, shifting the modes at 1260 and 1143 cm$^{-1}$.

At low temperature, TMA can desorb from the C=O∙∙∙Al(CH$_3$)$_3$ complex, as shown in the QCM results, and subsequent water exposure leads to relatively small changes in the starting PMMA. At high temperature, the physisorbed structure converts to form a PMMA–TMA reaction product with a characteristic vibration at 1568 cm$^{-1}$. This reaction proceeds relatively rapidly at higher temperature (within the first 10 TMA doses at 150 °C, Figure 2.3). Moreover, the PMMA–TMA reaction product is stable; the IR data in Figure 2.1 shows that it remains present after water exposure.

The QCM results (Fig. 2.2) also show TMA uptake in the PMMA bulk, followed by desorption. QCM analysis on a planar quartz crystal substrate typically shows a mass uptake of ~40 ng cm$^{-2}$ per cycle during steady-state TMA–water$^{121,122}$ We find that at 100°C, one TMA dose onto
PMMA produces a mass change $> 500 \text{ ng cm}^{-2}$. This large mass change is ascribed to TMA diffusing into the polymer bulk. This sub-surface diffusion of ALD precursors into several different polymers has been previously observed by IR, QCM, TEM and other methods\textsuperscript{33,99–105}. We also note that the magnitude of overall mass uptake after 150 TMA doses (Fig 6.2a) increases with temperature between 70 and 100°C, but it is not as large at 140°C. At low temperature, where IR indicates that TMA does not react with PMMA, increasing the temperature will promote TMA diffusion. At high-temperature, the covalent reaction between TMA and PMMA will fix TMA and could slow sub-surface diffusion. Therefore, the temperature-dependent QCM results further support the TMA adsorption–desorption and reaction mechanism described above.

We now consider possible PMMA–TMA reaction products that could account for the observed data, including the characteristic IR vibration at 1568 cm\textsuperscript{-1}. One product we considered is structure A in Figure 2.4. This could be produced by breaking the carbonyl to form Al-O, transferring a methyl to the carbon. Even though this product was previously considered\textsuperscript{107} this reaction scheme is not likely because methyl migration products from TMA adducts typically require stronger electron donating functional groups than ester carbonyls to adequately destabilize the aluminum complex\textsuperscript{123}. Even so, we performed a geometry optimization for this structure and examined its vibrational modes using Gaussian, the resulting spectrum is plotted in Figure 2.4. The carbonyl stretch is lost and new C-O coupled modes appear between 1000 and 1300 cm\textsuperscript{-1}. No new modes appear in the 1500-1700 cm\textsuperscript{-1} region where the stable product is experimentally observed. Since this product lacks the vibrational signature of the resulting product, and because the reaction scheme is not favorable, we looked for other possible stable products.

Another possibility is that the TMA in the coordination complex interacts with the neighboring methoxy group in a pericyclic reaction to yield a covalent bond between Al(CH\textsubscript{3})\textsubscript{2} and the oxygen originally in the carbonyl. The methyl lost from the TMA reacts with the methoxy moiety forming ethane vapor and an ester carbonyl. The proximal Al center would then coordinate to the carbonyl oxygen in the same ester, as shown in product B, or in a neighboring
ester as shown in product C and in Figure 2.6. The reaction scheme in Figure 2.6 is energetically driven by formation of the strong Al-O and ethane bonds. The scheme is consistent with the relative stability of TMA reacting with esters, where TMA/ester complexes are known to catalyze Tischenko-like reduction of beta-ketoesters without degradation of the ester moiety. These pericyclic reactions are common in organic/inorganic complex formation and in other synthetic routes where TMA is able to promote methyl translation to form C-C bonds. Since products B and C are synthetically feasible, we considered them further for \textit{ab initio} modeling. Figure 2.4 shows the minimized structures and characteristic vibrational spectra. Both products B and C show loss of the C=O and coupled C-O stretching in the starting PMMA. Most notably, new modes appear in the 1500 -1700 cm\textsuperscript{-1} region that correspond to C=O stretching modes shifted to lower frequency due to Al coordination and near-neighbor bonding. In product B, the model constrains the Al coordination and bonding on the same ester unit and modes appear at 1520 and 1480 cm\textsuperscript{-1}. For product C, Al binds to adjacent esters, producing a C=O stretch at 1605 cm\textsuperscript{-1}. In highly TMA-saturated PMMA, further reactions between -O-Al(CH\textsubscript{3})\textsubscript{2} and other esters would form additional bond/coordination pairs, leading to further mode shifting. Based on this result, we believe that the mode at 1568 cm\textsuperscript{-1} arises from C=O stretching vibrations where the carbonyl carbon is also linked to -O-Al(CH\textsubscript{3})\textsubscript{2} (or possibly -O-Al(CH\textsubscript{3})-O-) with the Al further coordinated with oxygen on other chains, as depicted in Figure 2.6. To further support this scheme, we note that PMMA powder, which appears white as received, remains visibly unchanged after TMA exposure at low temperature, whereas TMA exposure at high temperature leads to a dramatic, visible color change to black that transitions to orange upon exposure to air. This coloration may therefore be due to products where the metal atom bridges multiple organic groups, as commonly found in metal-complexed dyes. The color shift could be attributed to atmospheric water slightly altering the metal-organic structure; the permanence of this color change attests to the stability of this final product.
2.5 Summary

Combining evidence from in situ infrared transmission and quartz crystal microbalance experiments with ab initio modeling analysis, we conclude that exposing TMA vapor to PMMA thin films leads to significant subsurface TMA infiltration where the TMA physisorbs onto C=O forming a metastable C=O⋯Al(CH₃)₃ structure. Upon TMA physisorption, the PMMA C=O stretching frequency at 1732 cm⁻¹ shifts to 1670 cm⁻¹ when the TMA Lewis base withdraws electrons from the C=O bond. At low temperatures (up to ~100°C), the interaction does not and the TMA readily desorbs. At higher temperatures an IR feature near 1568 cm⁻¹ arises, likely due to C=O stretching in resonant C=O⋯Al-O-C units. These bonds could possibly form through a pericyclic reaction involving a PMMA methoxy group interacting with a neighboring TMA stabilized through coordination with the C=O ester. This improved understanding of the mechanisms for TMA interaction with PMMA will also extend to other polymers and other Lewis-acidic metal-vapor reactants, helping to create knowledge and advance applications for sequential vapor infiltration processes.

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CHAPTER 3. Wafer-Scale Selective-Area Deposition of Nanoscale Metal Oxide Features using Vapor Saturation into Patterned Poly(methyl methacrylate) Templates

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3.1 Introduction

Creating and aligning nanoscale patterns in thin film materials is critically important for manufacturing reliable semiconductor devices. As feature sizes in silicon technology approach, and scale below 10 nm, new methods are needed to pattern, and align contact vias, lines, line cuts and other device features. Non-traditional methods, including directed self-assembly,8–10 multiple e-beam direct write systems,11 and nanoimprint patterning,12–14 are currently being investigated to define and position feature elements.

Atomic layer deposition (ALD) is a high volume manufacturing process used to deposit well controlled, nanoscale, high quality, ultra-conformal thin films. Recently, more groups are working to understand growth initiation reactions during ALD to chemically tune interface bonding, and achieve substrate-selective thin film growth15–20 where vapor precursors react only at desired locations to directly produce a patterned thin film. The ability to form patterns through vapor processes provides a distinct advantage over conventional methods. For example, patterning could be done with fewer lithography steps, reducing the need for harsh etch solution chemistry. However, achieving reliable vapor–based selective deposition at low temperature suitable for manufacturing requires a more basic understanding of selectivity in surface chemical attraction and bonding.

Compared to chemical vapor deposition (CVD), ALD enables better control of film thickness and conformality. Currently, most functional selective deposition processes are based on CVD reaction chemistry.21,22 Frequently, polymer films and self-assembled monolayers are used to
prevent or block nucleation,\textsuperscript{15–18} and in other cases monolayers act to ‘seed’ ALD.\textsuperscript{32} However, reactive ALD precursors will often diffuse and react within organic blocking layers,\textsuperscript{17,36–39} leading to unwanted nucleation and growth in the protected region.

![Figure 3.1. Schematic of a polymer film saturated by vapor infiltration followed by oxidation. The nano-patterned polymer is saturated with a precursor through extended exposure. Once infiltrated, the sample is annealed to remove the polymer, and oxidize the precursor to create a nano-patterned dielectric material that precisely mimics the starting pattern across a large surface area.](image)

In other studies, researchers have taken advantage of ALD precursor diffusion into polymers\textsuperscript{36} to improve materials or create new structures. Lee et al. found that exposing spider silk to extended exposures of diethylzinc vapor led to an increase in mechanical fiber toughness.\textsuperscript{127} Similar treatments also improve tensile strength and elastic modulus of cellulose.\textsuperscript{128} Trimethylaluminum (TMA) infusion during Al\textsubscript{2}O\textsubscript{3} ALD on nylon also affects the modulus and hardness of the oxide coating.\textsuperscript{129} Infusing TMA into polyester fibers and annealing can create metal oxide fibers with controlled porosity.\textsuperscript{130} Processes and applications of TMA infusion and reaction into PMMA are well known from several previous studies. Tseng et al.\textsuperscript{131,132} explored TMA/water infusion into high aspect–ratio PMMA features to add physical reinforcement and prevent feature collapse. Using PMMA–containing block co-polymers, TMA can diffuse and react selectively within the PMMA block, creating oxide nano-features after oxidation.\textsuperscript{133–135} While these vapor infusion processes have been given several names,\textsuperscript{127,128,130,133} all use extended vapor exposures of metal–containing reactants to penetrate
and either directly bond, form a complex, or become entrapped within the polymer bulk. Studies of detailed reaction mechanisms and reactant exposure dose have mostly focused on the case of TMA infusion into PMMA.\textsuperscript{38,136,39,137} In this case, at moderate temperature, TMA forms an adduct with the PMMA ester group followed by reaction to form C–O–Al(methyl) units.

In this work, we demonstrate that polymer thin films patterned with a variety of nano- to cm-scale features across wafer-scale dimensions absorb and accumulate relatively large amounts of ALD precursor reactants, eventually leading to adsorption saturation. After saturation, an anneal step converts the polymer layer to a metal oxide layer that accurately reproduces the original pattern shape, thereby achieving selective-area metal oxide film formation on surface regions originally covered with polymer. The resulting metal oxides are not fully dense layers, but rather they show porosity that depends on the detailed post-infiltration anneal conditions.

The procedure developed is shown schematically in Figure 3.1. We refer to the extended vapor exposure step as saturated infiltration. We use a dry ALD reactor to expose a patterned polymer to multiple doses of one reactant, (e.g. TMA) where each dose consists of one or more exposure, each followed by a ‘hold’ step to lengthen precursor exposure, followed by an inert gas purge. These dose cycles are repeated several times to allow the reactant to diffuse and interact with the reactive sites within the polymer film bulk.\textsuperscript{38,39} The saturated polymer is then exposed to water for one second before being exposed to water vapor in the lab ambient. This process uses the basic understanding of polymer/precursor interaction chemistry\textsuperscript{38} to ‘seed’ the conversion of the polymer to metal oxide. The extent of precursor reaction in the polymer allows full reaction saturation to completely reproduce features of different sizes on a single substrate. Likewise, for uniform starting features, the extent of exposure can be used to adjust and shrink the final feature width to less than the starting pattern. We use poly (methyl methacrylate) (PMMA), a common semiconductor resist,\textsuperscript{52} patterned by electron beam lithography. Since the process uses long TMA exposures followed by one water dose, substrate regions not covered by polymer receive only one formal ALD cycle limiting growth
to the single-monolayer regime (i.e. a few Ångstroms thick).53

In this method the patterned polymer is a positive template for the resulting metal oxide, and therefore is chemically opposite of the more common negative pattern generated with organic blocking layers. Since precursor adsorption is self-limiting within the polymer bulk, a wide range of metal oxide feature sizes can be produced simultaneously across large surface areas. We demonstrate films with uniform thickness from less than 10 nm to over 150 nm (limited only by process time), and patterned features greater than 1 cm to as small as 20 nm (limited by available polymer patterning tools). Results also show reliable and repeatable pattern generation across a 150 mm silicon wafer (limited by reactor size). We also extend this technique to pattern additional materials by matching polymer reactivity with other vapor reactants.

3.2 Results and Discussion

3.2.1 Polymer Saturation and Metal Oxide Thickness

To produce patterned metal oxide films, PMMA was exposed to a sequence of TMA dose/hold/purge steps to attain full TMA saturation within the polymer, followed by a single water dose. The saturated polymer was then calcined at high temperature (i.e. 500 °C) in air to form the metal oxide and remove the polymer. In this article we use “TMA dose” and “TMA exposure” to indicate the number of TMA dose/hold/purge steps collectively completed before a single one second water exposure. Using several dose/hold/purge steps allow the reactant partial pressure to be periodically refreshed in the reactor, thereby promoting reactant transport into the polymer film. While single long TMA exposure step may enable a similar outcome, it would require large amounts of precursor to be dosed continuously while saturation occurs.

The thickness of the resulting metal oxide, as formed after infiltration and annealing, was governed by the thickness of the starting PMMA and the number of TMA dose steps. Figure 3.2a and b show alumina thickness, determined by ellipsometry, with respect to the number of TMA doses at 90 °C for starting PMMA layers of 60±0.5 nm, and 500±0.5 nm on silicon, respectively. After exposure, all samples were annealed in air at 500 °C for 30 minutes.
the 60 nm PMMA layer, the resulting metal oxide film thickness depended strongly on TMA exposure during the first 50-100 TMA doses then saturated at approximately 18 nm after 100 doses. Using 500 nm of PMMA, the resulting oxide thickness saturated after about 250 TMA doses, producing a metal oxide film 155 nm thick. The saturation curves are analogous to those produced for ALD precursor exposures, indicating controlled, saturated, and self-limiting film growth. In this case the polymer bulk provides sites for reactant adsorption. Saturation also leads to predictable scaling of the resulting metal oxide thickness, discussed below.

Figure 3.2c shows the influence of hold time (between 0 and 300 seconds) on the resulting metal oxide thickness for 60 nm of PMMA exposed to 5 or 100 TMA doses. Longer hold times led to thicker oxide films, even for very few dose steps. After only 5 TMA doses the sample did not reach the saturated thickness value, but longer hold time increased the film thickness. Increasing the purge time led to a thickness decrease (Figure 3.2d). Very long purge times (>180 s/dose) enabled oxide formation, indicating TMA retention within the PMMA. We find the saturated infiltration process does not require the purge between TMA doses. Figure 3.2e shows aluminum oxide thickness versus number of TMA doses using 0 and 30 second purge steps. Without the purge the film thickness saturated after only 5 doses. Even though faster saturation occurred without a purge, films produced with a 5–30 s purge step were more reproducible, likely because the purge helped promote TMA motion and uniform distribution with the polymer film.
Figure 3.2. Saturated infiltration process parameters. Lines are meant to guide the eyes. Recipe: dose(hold)/purge, time in seconds, X is the variable being changed. All samples annealed at 500 °C. A) Increasing TMA doses leads to a saturating alumina thickness after 100 TMA doses. B) Thick alumina films can be achieved while maintaining a non-growth surface. C) Altering the hold time of TMA in the reactor led to increased growth per dose. D) Altering the purge time of each TMA dose led to decreased growth per dose. E) By removing the purge from each TMA dose thick films were grown rapidly but with increased variability. F) In situ infrared spectra show polymer saturation after 50 TMA doses, the formation of a Lewis-acid base adduct between the PMMA ester and TMA at 1670 cm⁻¹, and the partial reversion of this adduct after water exposure (scans shown in differential form show the difference of one spectra from the one below it).

The trends in Figure 3.2 indicate that TMA diffuses into the PMMA during the dose/hold step (promoting thicker oxide film growth), but partially diffuses out of the polymer during the purge step. Previous in situ infrared spectroscopy (IR) and quartz crystal microbalance
analyses of the interaction between TMA and PMMA\textsuperscript{38,39} show that at low temperature, TMA diffuses into PMMA to form a Lewis acid/base adduct with the PMMA ester moiety. Figure 3.2f shows representative differential \textit{in situ} IR spectra collected when 170 nm thick PMMA was exposed to TMA at 90 °C. The loss of the C=O peak at 1732 cm\textsuperscript{-1} and the corresponding gain at 1670 cm\textsuperscript{-1} are consistent with quantum chemical vibrational calculations for C=O…:Al(CH\textsubscript{3})\textsubscript{3} Lewis acid/base adduct formation.\textsuperscript{38,138} The differential spectra show a large change during the initial TMA doses with no observed changes after 50 TMA doses, indicating polymer saturation after about 50 TMA exposures. After water exposure, the IR shows partial return of the C=O peak, consistent with Al-O bond formation.\textsuperscript{38} Therefore, overall trends in Figure 3.2 are ascribed to TMA diffusing into and interacting with polymer ester sites until all sites within the polymer are saturated. The water dose and anneal steps then convert the organic/aluminum layer into an aluminum oxide film.

The TMA infusion also produces some physically swelling of the polymer. Profilometry analysis of patterned 300 nm thick PMMA films before and after TMA saturation at 80-100 °C showed a 30% fractional thickness increase, with no detected lateral expansion.
Figure 3.3. XTEM images of starting PMMA and resulting alumina films formed at 90 °C. A) Patterned PMMA 30 nm thick. B) 100 TMA doses into 60 nm PMMA led to 19 nm of alumina following the anneal step, whereas the region without PMMA shows only native oxide present. Film thicknesses are consistent with ellipsometry in Figure 3.2. In the TEM images, the variable contrast in the deposited metal oxide layer is consistent with uniform film porosity.

3.2.2 Patterning and Area Selectivity

Figure 3.2b shows ellipsometry values collected in regions of the substrate that were originally covered only with native silicon oxide. Using ellipsometry, the starting native oxide was 1.8±0.2 nm thick. After anneal, without exposure to TMA, ellipsometry typically showed a slight increase in oxide thickness to 2.0±0.2 nm. The saturating infiltration process (many TMA exposures followed by one water step) is formally similar to a single TMA/water ALD cycle; minimal oxide growth is expected in regions not covered by PMMA. On surfaces covered with native silicon oxide, the resulting total oxide thickness (silicon oxide and aluminum oxide) was 2.5-3.0 nm. Total oxide thickness was not observed to increase from 50 to 800 TMA doses, the highest number of doses tested. The net increase of 0.5-1 nm was more than the expected thickness change (0.11 nm) during steady state TMA/water ALD, but it is consistent with previous studies showing increased aluminum oxide growth for long TMA exposure steps.53
Figure 3.4. XPS data shows the elemental differences between the growth (PMMA) and non-growth (silicon) regions of a 2 cm² silicon substrate that was half coated with 500 nm of PMMA. The sample was infiltrated with 500 TMA doses at 90 °C and annealed at 500 °C. Significantly less alumina was seen on the non-growth region. Following a 1 hour 0.01 M nitric acid etch there was no detectable alumina on the non-growth region while the growth region remained unchanged.

Cross-sectional TEM images collected from two different patterned samples, before and after, 100 TMA doses at 90 °C on a 500 nm-wide PMMA feature are shown in Figure 3.3. The PMMA in Figure 3.3a is 30 nm thick in the feature center and tapers toward the edge. This shape is well reproduced in the resulting Al₂O₃. The Al₂O₃ feature shows a thin native silicon oxide layer covered with roughly 19 nm of uniformly porous aluminum oxide. The final thickness is consistent with data in Figure 3.2a for a starting PMMA thickness of 60 nm. At the scale of the TEM images, no aluminum oxide can be detected on the native oxide that was not covered by PMMA. For this and all other samples analyzed, the film thickness observed in TEM was consistent with thicknesses obtained from ellipsometry.

Figure 3.4 shows O 1s, Si 2p, and Al 2p XPS traces collected from the “growth” (PMMA coated) and “non-growth” (bare silicon) regions of the substrate. For this experiment, we infiltrated a patterned 500 nm thick PMMA film at 90 °C with 500 TMA doses and annealed at 500 °C for 30 min. The growth region showed a strong Al 2p peak near 74.6 eV with a very weak Si 2p peak at 100.4 eV from the silicon substrate, indicating a thick Al₂O₃ layer. In the non-growth region, the Si 2p signal from the substrate was prominent whereas the Al 2p peak was much weaker, indicating a thin aluminum oxide monolayer, which is consistent with the above ellipsometry results. After the sample was immersed in a 0.01 M nitric acid solution for 60 minutes, the Al 2p signal disappeared from the non-growth region, whereas changes in the intensity of the Al 2p peak in the growth region were not detected. Ellipsometry in the growth region shows some increase in film porosity. This shows that complete selectivity (within the 0.5% atomic sensitivity of XPS) can be achieved on patterned samples using saturated vapor infiltration. As discussed below, XPS analysis showed no difference in carbon content in areas with metal oxide vs regions without metal oxide.
3.2.3  Metal Oxide Film Composition, and Extent of TMA Adsorption in PMMA

Film composition was examined by XPS and EDX, and refractive index was analyzed using ellipsometry. Films produced under well controlled conditions, i.e. TMA saturation at 90 °C followed by anneal at 500 °C for 30 minutes, the Al 2p and O 1s XPS spectra in Figure 3.4 were consistent with Al₂O₃, with some C 1s signal also present. Quantitative analysis of carbon content is inhibited by adventitious carbon present on all films. However, the C 1s signal intensity measured on PMMA–template oxide films formed at controlled conditions are the same as those observed by XPS on silicon oxide after the same TMA exposure with no PMMA present, indicating the carbon content in the oxide bulk was near, or at the detection level of XPS, ~0.5 at%. When TMA exposure is done at higher temperature, or when the calcination step is performed at <500 °C, or for less than 30 min, the resulting films show a larger C 1s signal, consistent with carbon in the metal oxide bulk.

Using a known starting PMMA layer thickness, the resulting metal oxide film thickness and density can be used to estimate the fraction of ester groups in PMMA that bind to TMA during exposure saturation. For a given PMMA thickness, the number of ester groups per unit substrate area can be found from the PMMA density, 1.18 g/cm³, and monomer molecular weight, 101 g/mol. Using this with the known density of ALD Al₂O₃ deposited at 90°C, (density ≈ 2.7 g/cm³),[36] we find that under full ester–site saturation (i.e. one TMA per ester site), the Al₂O₃:PMMA thickness ratio would be approximately 0.22. In Figure 3.2, PMMA layers of 60 and 500 nm produced Al₂O₃ layers 18 and 155 nm, respectively, giving a somewhat larger Al₂O₃:PMMA ratio of approximately 0.30. This could result from more than one TMA per ester site, or from film porosity in the resulting metal oxide film.

For TMA–saturated PMMA annealed for 30 min in air at 500 °C, ellipsometry analysis of the resulting oxide gives $n_{\text{eff}} = 1.389$ at 633 nm, which is less than the $n = 1.630$ that was measured in our lab (and by others)\textsuperscript{1,139} for ALD Al₂O₃ formed using TMA/water at 100 °C and annealed at 500 °C. This indicates porosity in the oxide layer formed from PMMA infusion, consistent with TEM in Figure 3.3. Using the series effective medium approximation,\textsuperscript{140–142} we estimate the porosity (i.e. void fraction in film volume ) is 0.27. As discussed above, converting a fully-
saturated PMMA layer to dense Al₂O₃ will give an Al₂O₃:PMMA thickness ratio 0.22, whereas the observed ratio was 0.3. Dividing the 0.22 thickness ratio by 0.73 (i.e. the measured fraction of filled space = 1 - 0.27), gives a revised expected thickness ratio of 0.30, the same as found experimentally. This analysis further confirms that TMA saturation coincides with a 1:1 ratio of TMA:esters in the PMMA.

3.2.4 Effect of Infiltration Temperature on Vapor/Polymer Interaction and Saturation

Results regarding the temperature influence on oxide film growth between 45 and 150 °C are shown in Figure 3.5. Figure 3.5a demonstrates that increasing the temperature from 45 to 90 °C leads to more rapid growth (i.e. more rapid TMA uptake) during initial TMA doses. Lower temperatures led to somewhat thicker films, but the film was less dense than films formed at 90 °C, as indicated by a lower refractive index. Increasing the temperature further to 150 °C led to slower film growth, consistent with impeded TMA incorporation.³⁸

Under saturating infiltration conditions at 90 °C a 500 nm PMMA film produced an oxide layer of 155±2 nm. Figure 3.5c shows that the final alumina film thickness scales linearly with the starting PMMA thickness. At higher temperature, where TMA saturation was not achieved, alumina thickness also increased linearly with PMMA thickness, but a thinner oxide was produced. This linear relationship is consistent with TMA infiltration throughout the polymer bulk as opposed to in near surface regions. Also shown in Figure 3.5c are results obtained when the single vapor dose/hold sequence was replaced by a more typical dual vapor, TMA/water, ALD-type dosing cycle (1 cycle: TMA(hold)purge/water(hold)purge, 1(60)180/1(60)180) at 90 °C followed by the same post-process anneal in air at 500 °C. All PMMA thicknesses led to a fixed oxide thickness. This indicates that the water exposure after TMA forms an encapsulating alumina layer that blocks penetration throughout the polymer bulk.¹⁴³ As shown below, repeated TMA/water cycles on thicker PMMA produced films that cracked and peeled from the surface, whereas saturated TMA exposure led to well-formed stable films.
Figure 3.5. The effect of exposure temperature on alumina thickness. Lines meant to guide the eyes. Recipe: Dose(hold)/purge x number of repeated doses, time in seconds. All samples annealed at 500 °C unless otherwise noted. A) Increased TMA exposure caused alumina saturation. B) Zoomed in view of Figure 3.5a highlights up to 50 TMA doses. C) A linear relationship between PMMA thickness and alumina thickness was observed. Exposure at 90 °C consistently produced films thicker than at 150 °C after 200 TMA doses. When dosing alternating between TMA, and water without full reactant saturation, no PMMA thickness dependence was observed. D) The infiltration temperature was altered from 45 to 150 °C using both 5 and 200 TMA doses. Temperatures higher than the glass transition temperature (Tg = 95 °C) show thinner films in both cases, whereas the low temperature films grew thicker with increased TMA dosing. E) Increased annealing temperature and time led to increased alumina refractive index, directly proportional to density.

Figure 3.5d provides further insight into the role of temperature in metal oxide film formation. For this experiment, 130 nm of PMMA was exposed to 5 or 200 TMA doses at various temperatures. Between 60 and 90 °C, 200 TMA doses saturated the PMMA to produce 55-58 nm of metal oxide. Increasing the temperature over 90 °C led to a decrease in net film growth, indicating less TMA diffused in and/or was retained in the PMMA. Using 5 TMA doses led to thinner oxide films, with the thickest film formed near 80-90 °C. From these results, the fastest infiltration rate occurs at a critical point, 80-90 °C. For TMA infiltration into PMMA, we note that the critical temperature is close to the reported PMMA glass transition temperature, Tg = 95 °C. Previous analysis of ALD precursor infiltration into polymers suggests fastest penetration near the Tg. At temperatures exceeding 90 °C, IR analysis shows that TMA can further react with PMMA during exposure to form Al-O bonds that can potentially crosslink the polymer chains, leading to inhibited TMA infusion by physically blocking diffusion and/or by increasing the polymer Tg.

### 3.2.5 Film Density vs Anneal and Post–Processing Treatment

Results in Figure 3.5e show that increasing the post-exposure anneal time and temperature produces higher density metal oxide. We also find that oxide density is influenced by the molecular weight of the starting polymer. Using PMMA with a lower MW (15,000 g/mol) helped enhance the density somewhat (n_eff = 1.396, porosity = 26 %) whereas higher MW (350,000 g/mol) produced less dense oxide layers (n_eff = 1.374, porosity = 29 %). Increasing
the anneal temperature and duration tended to increase the refractive index and density, Figure 3.5e.

Using the Al$_2$O$_3$ films formed with PMMA infiltration and annealing, we applied a TMA/water ALD sequence post anneal to increase the overall film density. We found that applying one TMA/water ALD cycle increased the refractive index from 1.39 to 1.43, and after 15 ALD cycles, the index was 1.58, i.e. about 5% porosity, demonstrating significant post-process film densification.
PMMA was patterned with an electron beam, saturated with TMA and then annealed to alumina. a,b) SEM micrographs of patterned PMMA and the resulting alumina pattern, respectively, show high fidelity in pattern transfer. c) SEM micrograph of patterned PMMA, top, and the resulting alumina, bottom, show the pattern shrinking 33% from 30 to 20 nm following sub-saturating infiltration and anneal. d) Varying the TMA dose controlled the width of the patterned alumina. The original PMMA feature width was 188 nm. e,f) SEM of patterned alumina. g) Side view of alumina film on silicon. h) SEM micrograph of patterned titanium dioxide. In all plan view micrographs patterns through to the silicon substrate are the lighter color except (c) where charging of the nonconductive alumina caused it to appear lighter. i) A 300 nm wide PMMA line (200 nm thick) treated with 200 TMA/water ALD cycles with extended exposures (left) and 200 TMA doses (right).

3.2.6 Selectivity Scaling and Large-Area Uniformity

Figure 3.6 shows that selective TMA infiltration into patterned PMMA enables well-defined metal oxide features with a wide process latitude. Figure 3.6a and b show micron-scale PMMA patterns and the resulting aluminum oxide, respectively, and Figures 3.6a-f show the pattern versatility, including PMMA lines as small as 30 nm (Figure 3.6c, top) leading to 20 nm alumina lines (6c, bottom) using a sub–saturating TMA exposure. Starting with a 188 nm wide PMMA line, Figure 3.6d shows feature width reduction was adjusted by the extent of PMMA saturation. After 10, 50 and 100 TMA doses at 90 °C, the lines were 149, 180 and 188 nm wide, respectively. The effect of line shrinking on pattern registry was not directly characterized. The ability to create features smaller than the starting patterned may allow features smaller than the limit of optical or physical patterning. A cross–sectional SEM image in Figure 3.6g shows uniform aluminum oxide on silicon.

To show versatility of the saturating infiltration approach, titanium oxide patterns were formed at 150 °C using titanium tetrachloride (TiCl₄) infiltration into PMMA, Figure 3.6h. The dosing scheme was the same as that used for TMA. The darker regions correspond to the titanium oxide lines.
Figure 3.7. A 150 mm wafer was patterned using electron beam lithography to determine process scalability. A 200 by 200 μm pattern was applied in groups of 4 as a 4 x 4 matrix across the wafer. A) SEM micrograph of representative patterns at each position on the wafer. B) Photograph of the pre-patterned PMMA coated wafer with symbols displaying approximate pattern locations. C) The width of the dark, alumina, portion of the diamond in the lower left, and the 2nd and 5th circles from the center of each micrograph were measured at each location.
The importance of polymer infusion throughout the polymer bulk is highlighted in Figure 3.6i. Starting with a 200 nm thick patterned PMMA layer, the image on the right shows a well-defined patterned oxide film produced by saturating PMMA with 200 TMA doses at 90 °C. However, if we used a more traditional TMA/water ALD-type cycle, as described in the previous section, the oxide cracks and peels from the substrate. This is likely due to the reaction between TMA/water limiting TMA penetration into the polymer bulk leaving unreacted PMMA at the substrate surface. Exposing polystyrene/PMMA block co-polymers with nanoscale PMMA regions to TMA/water doses can successfully produce a patterned metal oxide that mimics the PMMA shape. In those studies, the TMA dose time may be sufficient to saturate the reactant within the small PMMA volume.

The saturated exposure achieved in the vapor infiltration process is also important to control growth selectivity. While substrate selectivity is difficult to quantify, Figure 3.2b shows a selective thickness ratio of 155:1 nm, which is significantly larger than other low-temperature selective deposition methods. A selective chemical vapor deposition process for Co at 200 °C shows that selectivity degrades after 6 nm of Co growth. Using TMA/water infusion with PS-b-PMMA block copolymer templates, Peng et al. found alumina present in the PS region (where growth was not desired) after only 6 TMA/water cycles. Targeting and utilizing polymer/reactant saturation at low temperature allows thick metal oxide films to selectively form at pre-selected sites on the substrate with minimal growth on non-desired regions. The infiltration process is also rapid, the thickness of the alumina film was 4.9 nm on the growth region after one TMA/water cycle using 60 nm PMMA, nearly 50 times thicker than the standard 0.11 nm film expected after one TMA/water cycle in a typical ALD process.

As shown in Figure 3.7, this saturated vapor-infusion patterning technique extends to wafer-scale dimensions while maintaining high fidelity. Using 150 nm thick PMMA patterned on a 150 mm diameter silicon wafer, we formed 200×200 μm² patterned areas (partially shown in Figure 3.6a and b) in groups of 4 at 16 locations (i.e. 64 patterned sites) distributed across the wafer. The wafer was then exposed to saturating vapor conditions at 90 °C, followed by a 30 min anneal at 500 °C. Figure 3.7a shows images of part of the pattern from each site. The line
width for several features at each location, collected using ImageJ software, were plotted in Figure 3.7c, showing that the metal oxide was selectively patterned uniformly and consistently across the 150 mm wafer. By analyzing PMMA features on similar samples, we found that for the e-beam patterning tool we used, feature size increased by a few percent laterally across the 150 mm wafer. This trend was faithfully reproduced in the metal oxide feature size observed.

3.3 Conclusion

By controlling metal-containing vapor reactant infiltration and saturation into blanket and pre-patterned polymer templates, we show reproducible selective-area metal oxide thin film formation with nanoscale feature dimensions, excellent process uniformity and pattern selectivity over wafer-scale surface areas. The resulting oxide films were more porous than directly deposited ALD metal oxides, but the porosity can be adjusted and controlled by the conditions for post-exposure anneal and by post-formation application of ALD. By understanding reactant absorption and saturation within a polymer, the saturating infiltration process enables scalability to near limitless feature size, shape and film thicknesses, with good uniformity across large sample areas. Due to reactant saturation, a non-uniform vapor exposure will produce uniformly thick films, as long as the area of least exposure is sufficient for saturation. Using controlled sub-saturating exposures, we find it is also possible to shrink and adjust patterned feature sizes, further pushing the possible size range of chemical surface patterning. In addition to thin films, PMMA, and other reactive polymers, can be patterned in 3D to create steps, bowls, pyramids, lenses, and other objects. Based on the process scaling shown above, we expect that the process shown here would readily adapt to 3D structure formation.

Overall, the patterning method demonstrated here could prove useful, for example, for selective dielectric deposition for advanced electronics, chemical patterning of electronic isolation or contacts, directed placement of porous low-k dielectrics, membrane and chemical separations applications, and to form permeable films with predetermined thickness and porosity.
3.4 Experimental Section

3.4.1 Chemicals and Materials

Supplies were purchased commercially and used as received. Poly(methyl methacrylate) (Fluka Analytical, MW 97,000) was chosen because it can be patterned, has reactive groups, and a large free volume. Trimethylaluminum (Strem Chemicals, min 98 % pure) was used due to its prevalence in the literature, small molecular size, and high reactivity. TMA was co-reacted with deionized water. The reactor was purged with high purity nitrogen gas (Machine & Welding Supply Co) that was further purified with an Entegris GateKeeper located directly upstream from the reactor input. PMMA was spun cast onto silicon substrates (University Wafers, P-type, <100>).

Spun cast films were created by first dissolving PMMA into toluene (Fischer Scientific) from one to eight percent by weight PMMA. Silicon wafers were cleaned with acetone (Fischer Scientific) and dried by spinning at 2000 rpm for one minute. The surface of the silicon was then flooded with PMMA solution and then spun at 2000 rpm for one minute. The sample was then heated on a hot plate (Isotemp, Fisher Scientific) at 200 °C for two minutes to remove toluene.

3.4.2 Polymer Patterning

Patterning was accomplished using a Raith 150 TWO electron beam lithography system. Test samples were used to determine proper dosing times for varying thicknesses of PMMA. For example, 200 nm PMMA was patterned with an area dose of 180 μC/cm² and a line dose of 900 pC. Patterns were developed in 1:3 isopropyl alcohol: methyl isobutyl ketone (Acros) for 55 seconds and then isopropyl alcohol alone for 30 additional seconds. PMMA acts as a positive photoresist where the areas exposed to the electron beam are removed. The Raith system is available through North Carolina State University’s Nanofabrication Facility and was used to pattern PMMA features as small as 30 nm.
3.4.3 Saturated Vapor Infiltration

The vapor infiltration, reaction and annealing approach is shown schematically in Figure 3.1. For the vapor infiltration process, we placed patterned PMMA thin film samples into a custom made, viscous-flow, hot-walled, vacuum reactor.\textsuperscript{37,43,143} The vapor reactant (e.g. TMA or TiCl\textsubscript{4}) was introduced into the reactor and held by closing all ports into and out of the reactor for a set time period. The reactor pressure during vapor dosing was approximately 800 mTorr, and the temperature was fixed at values between 45 and 150 °C. The reactor was then purged with N\textsubscript{2}. In this article we use “TMA dose” to indicate the dose/hold/purge steps collectively. The dose/hold/purge sequence (i.e. one dose as it is referred to in this paper) was repeated \(n\) times followed by a single dose of water for 1 second at approximately 800 mTorr, followed by a final N\textsubscript{2} purge. A typical sequence was a 1 second TMA dose, 60 second hold and 30 second purge, repeated \(n\) times, followed by a 1 second water dose and 45 second nitrogen purge. This is denoted as \([(1(60)30) \times n + (1/45)]\). The final water dose/purge step helps to stabilize the reacted PMMA during subsequent exposure to ambient water vapor. For some experiments the spun-cast PMMA was subjected to a pre-bake in the reaction chamber at 150 °C for 30 min before infiltration to remove any water present in the PMMA. The metal oxide films tended to be slightly (< 10 \%) thinner with the pre-bake than without the pre-bake, suggesting some water was present in the PMMA after patterning. The films studied here were formed without the pre-bake step.

After vapor exposure, samples were removed from the reactor and transferred in air to a furnace (Thermoscientific, Thermolyne) to be annealed in air. Samples were heated quickly (20 °C/min) to 500 °C and held for 30 minutes, allowed to cool in the furnace to room temperature, then removed for analysis.

3.4.4 Characterization

The thickness of the starting PMMA and the resulting metal oxide were determined using an \(\alpha\)-SE ellipsometer (J.A. Woollam Co., Inc). For films > 100 nm, thickness was also measured using a Dektak 150 profilometer (Veeco). Profilometer measurements provided physical
thickness values that were then correlated with the ellipsometry readings. The thickness and refractive index were extracted from the ellipsometry results by fitting the data to a Cauchy model. Measurements were taken from 400 to 900 nm; refractive index values were reported at 633 nm. Cauchy coefficients for aluminum oxide were taken from literature stored in the Woollam software. Cauchy values for A ranged from approximately 1.34 to 1.45 and values for B ranged from approximately 0.003 to 0.0045.

Scanning electron microscopy (SEM) images of the starting patterned polymers were collected using the imaging electronics built into the Raith 150 TWO patterning system, and feature sizes were estimated using ImageJ 1.47 software. The patterned oxide layers were imaged using a JEOL JSM-6010LA SEM that was equipped with energy-dispersive X-ray spectroscopy (EDS) to identify the elemental composition of the annealed alumina films. For high resolution images, we used an FEI Verios 460L SEM.

We performed XPS analysis with an XPS Specs System with a PHOIBOS 150 analyzer. Al 2p, O 1s, and Si 2p spectra of all the samples were recorded with Al Kα X-rays (incident energy 1486.6 eV). The collected data was subsequently processed and modeled with CasaXPS software. The binding energies were calibrated to the O 1s and Si 2p peaks for the growth and non-growth surfaces, respectively.

3.5 Acknowledgements

The authors thank Sarah Atanasov for her assistance with XPS, the North Carolina State University Nanofabrication Facility for the use of their Raith 150 TWO system especially the tool manager Bruce Sprague, S.M.D. and Lam Research Corporation for support. The authors acknowledge the use of the Analytical Instrumentation Facility (AIF) at North Carolina State University, which is supported by the State of North Carolina and the National Science Foundation.
CHAPTER 4. Paired selective deposition for the co-patterning of two dielectrics from one patterned photoresist

Erinn C. Dandley, Jennifer Ovental, Gregory N. Parsons

This section is an article in preparation and may be revised for future publication.

4.1 Introduction

As Moore’s law pushes semiconductor manufacturers to create features at and below the 10 nm node, new techniques need to be investigated for patterning and aligning layers. To create the desired feature size photoresists are often subject to multiple exposures. This allows for the formation of features significantly below the limit of the 193 nm wavelength light currently being used by manufacturers. 149,150

To continue to shrink pattern size, new methods have been developed for deposition as well as etching. Atomic layer deposition (ALD) is a thin film technique for the deposition of metals and metal oxides with sub-nanometer thickness control. Techniques have been being developed for a little over a decade to advance ALD to deposit selectively only in areas of interest while leaving other areas uncoated. 35 Researchers have been using passivation layers, such as self-assembled monolayers or polymers, to deposit TiO₂, Ir, Pt, Ru, Al₂O₃, ZrO₂, HfO₂, AZO, ZnO, PbS, in areas lacking the passivation layer. Others have used patterned polymers or block co-polymers to serve as activating layers to promote the selective growth of TiO₂, Al₂O₃, ZnO, SiO₂, W within the polymer.

Despite advances in patterning smaller elements and selective deposition of materials, alignment of layers is challenging and of critical importance to make functional integrated circuits. The combination of shrinking feature sizes and increasing patterning steps has led to the need for pattern edge placement error to be in the single nanometer regime. 153,154
To our knowledge, the utilization of one patterned photoresist to selectively deposit two different materials, one in the photoresist region and one in the photoresist-free region, has not been studied. Many resources are devoted to making photoresist patterns; utilizing one patterned photoresist for the selective deposition of two materials would be highly beneficial. Depositing this way also removes an alignment step between the two deposited materials, as there is no need for a second resist layer to pattern the second material.

In this paper we discuss a method that utilizes differences in ALD precursor reactivity with the photoresist poly(methyl methacrylate) (PMMA). Previously, we determined the likely reaction mechanism between the common ALD precursor, trimethyl aluminum (TMA), and PMMA. We then went on to utilize the knowledge of this reaction to selectively deposit alumina by saturating patterned PMMA with TMA and then oxidizing to remove the polymer and convert TMA to alumina. Here, we take the process one step further by using the patterned PMMA to passivate silicon to first selectively deposit ZnO or TiO₂ using ALD and then infiltrate the polymer with TMA to selectively deposit alumina, Figure 4.1. Using this two-step process we can use one patterned photoresist to selectively deposit two different materials.
Figure 4.1. Paired selective deposition schematic. Patterned PMMA was used as a blocking layer to selectively deposit ZnO using DEZ/water ALD in this example. Following ALD, PMMA was infiltrated with TMA and then samples were annealed to remove the polymer and oxidize TMA to alumina.

4.2 Methods

4.2.1 Chemicals and Materials

Supplies were purchased commercially and used as received. Poly(methyl methacrylate) (Fluka Analytical, MW 305,000), trimethylaluminum (TMA), diethylzinc (DEZ) (Strem Chemicals, min 98% pure) and titanium tetraisopropoxide (TTIP) (Acros Organics, min 98% pure) were used as received. All precursors were co-reacted with deionized water during ALD. The reactor was purged with high purity nitrogen gas (Machine & Welding Supply Co) that was further purified with an Entegris GateKeeper located directly upstream from the reactor input. PMMA was spun cast onto silicon substrates (University Wafers, P-type, <100>). PMMA was spun cast using a method previously described.126

4.2.2 Polymer Patterning

Patterning was accomplished using a Raith 150 TWO electron beam lithography system. Roughly 200 nm thick PMMA was patterned with an area dose of 180 μC/cm² and a line dose of 900 pC. Patterns were developed in 1:3 isopropyl alcohol: methyl isobutyl ketone (Acros) for 55 seconds and then isopropyl alcohol alone for 5 additional seconds. The Raith system is available through the North Carolina State University Nanofabrication Facility.

4.2.3 Atomic Layer Deposition

ALD was performed using a custom made, viscous-flow, hot-walled, vacuum reactor.37,43,143 DEZ and water were co-reacted to form zinc oxide films at a temperature of 90 °C and a pressure of 900 mTorr. TTIP and water were co-reacted to form titanium oxide at a temperature of 160 °C and a pressure of 1.2 Torr. ALD was performed with longer than normal purging to minimize the interaction between the precursors and the PMMA film so as to selectively deposit on PMMA free regions of the silicon wafer.
4.2.4  **Vapor Infiltration**

The vapor infiltration technique was previously described by Dandley et al.\textsuperscript{126} Briefly, we placed samples back in the same reactor described above after the ALD step to infiltrate the areas without polymer. Samples could also be made without removing them from the reactor between the ALD and infiltration steps but we chose to remove them for ellipsometry measurements between steps. Once back in the reactor, samples were exposed to TMA for an extended period of time to promote precursor infiltration into the polymer, Figure 4.1. A typical dosing sequence was a 1 second TMA dose, 60 second hold and 30 second purge, repeated \( n \) times before being reacted with a 1 second water dose and 45 second nitrogen purge. This is denoted as \([((1(60)30) \times n + (1/45))\]. After TMA exposure, samples were removed from the reactor and transferred in air to a furnace (Thermoscientific, Thermolyne) and annealed in air. Samples were heated at 20 °C/min to 500 °C and held for 30 minutes, allowed to cool in the furnace to room temperature and then removed for analysis. This final step serves to remove the polymer and oxidize the TMA to alumina.

4.2.5  **Characterization**

The thickness of the PMMA and metal oxides were determined using an \( \square \)-SE ellipsometer (J.A. Woollam Co., Inc). Ellipsometry readings were validated using profilometry previously.\textsuperscript{126} The thickness and refractive index were extracted from the ellipsometry results by fitting the data to a Cauchy model.

Scanning electron microscopy (SEM) images of the starting patterned polymers as well as the resulting patterned oxides were collected using the imaging electronics built into the Raith 150 \textsuperscript{TWO} patterning system.

We performed XPS analysis with an XPS Specs System with a PHOIBOS 150 analyzer. In addition to a survey spectra, Al 2p, O 1s, C 1s and Si 2p spectra of all the samples were recorded with Al K\( \alpha \) X-rays (incident energy 1486.6 eV). Zn 2p and Ti 2p spectra were collected for the alumina-zinc oxide and alumina-titania samples, respectively. The collected data was
subsequently processed and modeled with CasaXPS software. The binding energies were calibrated to the C 1s peak for all samples.

4.3 Results and Discussion

4.3.1 Zinc oxide deposits selectively in PMMA-free regions

To pattern two materials using one patterned photoresist ALD was first performed and was deposited selectively in the photoresist-free regions. It is important to select ALD precursors that will not damage or react with the photoresist. In this case we used diethyl zinc (DEZ) and water to create patterned ZnO. Samples were coated using 300 cycles of ALD to deposit approximately 50 nm of ZnO. The DEZ did not interact with the PMMA during ALD and therefore did not block diffusion of TMA into the polymer during the infiltration step.

The absence of ZnO on the PMMA coated region was supported by XPS results in Figure 4.2 and Table 4.1 which shows no detectable Zn 2p in the alumina (previously PMMA coated) region. For the XPS analysis, 1 x 2 cm² Si wafers were first coated with 175 nm of PMMA and then approximately half of each wafer was cleaned of PMMA using acetone followed by isopropyl alcohol followed by water. These wafers were then exposed to the precursors of interest, in this case 300 cycles of DEZ/H₂O by ALD and then TMA by infiltration followed by an annealing step, as described above, to a final thickness of approximately 50 nm of both ZnO and alumina.

Selective deposition of ZnO via DEZ/water ALD was selected due to previous reports of selective deposition using polymer blocking layers. Selective deposition of ZnO using poly(vinyl pyrrolidone) (PVP) as a blocking layer was reported by Ellinger et al. with feature sizes in the tens of micrometers. ²⁹ It has also been shown that DEZ/water will selectively react with the polystyrene (PS) block of the UV irradiated block-co polymer PS-b-PMMA and not within the PMMA region with feature sizes around 24 nm. ⁴⁸
Figure 4.2. XPS results of co-patterned alumina and ZnO. Spectra are shown for the Al 2p and Zn 2p regions. Red lines are scans from the alumina (previously PMMA coated) region of the sample while black lines are from the ZnO (PMMA-free) region of the samples. Diagram included at the bottom for clarity.

4.3.1 Selective TMA infiltration into PMMA is not inhibited by prior ZnO/water ALD cycles

Previously we described an infiltration method that utilized patterned PMMA to pattern alumina from the nano to micro scale simultaneously across a 6 inch wafer. To achieve this we took advantage of the diffusion of TMA into PMMA and the subsequent reaction between the two. By using the polymer itself as a three dimensional co-reactant to TMA we were able
to fully saturate the polymer reactive ester sites while only depositing one monolayer of alumina on the polymer free silicon regions.\textsuperscript{126}

Table 4.1. XPS results for co-patterned sample with ZnO and Al\textsubscript{2}O\textsubscript{3} regions. Atomic percent rounded to the nearest whole number. $< 0.05 \%$ indicates the element was below the detection limit of the XPS.

<table>
<thead>
<tr>
<th></th>
<th>ZnO</th>
<th>Al\textsubscript{2}O\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>O 1s</td>
<td>55 %</td>
<td>60 %</td>
</tr>
<tr>
<td>C 1s</td>
<td>19 %</td>
<td>16 %</td>
</tr>
<tr>
<td>Zn 2p</td>
<td>26 %</td>
<td>$&lt; 0.5 %$</td>
</tr>
<tr>
<td>Al 2p</td>
<td>$&lt; 0.5 %$</td>
<td>22 %</td>
</tr>
<tr>
<td>Si 2p</td>
<td>$&lt; 0.5 %$</td>
<td>2 %</td>
</tr>
</tbody>
</table>

Because ZnO ALD did not interact with the patterned PMMA, there was no blocking layer to prevent the infiltration of TMA into PMMA. In this way, the patterning behaves exactly as before but with one key exception, the monolayer of alumina typically present on silicon regions was not present on the ZnO surface as measured by XPS, Figure 4.2. Upon further investigation we found reports of TMA etching ZnO during the deposition of alumina doped zinc oxide, a common transparent conductive oxide with applications in displays and photovoltaics.\textsuperscript{156,157} Although we did not notice a significant decrease in film thickness via ellipsometry on the ZnO region following TMA infiltration, it is possible that the decrease was below the detection limit of the ellipsometry measurement. The TMA monolayer could be absent due to a surface-cleaning mechanism where the TMA very slowly etched the ZnO instead of depositing a layer of alumina. Using this method to pattern ZnO and alumina, we were able to improve the selectivity of our previous process where we did not perform ALD before infiltration.\textsuperscript{126}
A representative SEM micrograph of co-patterned alumina and ZnO is shown in Figure 4.3. PMMA samples were patterned using electron beam lithography, treated with 300 DEZ/water ALD cycles followed by 300 TMA infiltration doses, and then annealed. Light areas in the images represent ZnO regions while the dark areas represent alumina regions. The three different images highlight co-patterning at various length scales.

Figure 4.3. SEM micrographs from ZnO-alumina co-patterned samples. Light areas are ZnO while dark areas are alumina.

Reactions between TMA and polymers have been previously reported and contributed to the selection of TMA as the precursor to infiltrate into and react with PMMA. TMA/water has been shown to interact within UV irradiated PS$^{48}$ and PMMA-b-PS.$^{4,47}$ Alumina ALD has been blocked by patterned PVP, with some alumina growth on the PVP but not beneath it.$^{25}$; a similar interaction was seen when PMMA was used as a blocking layer as alumina grew on the PMMA but not under it.$^{26}$

4.3.2 Titania deposits selectively in PMMA free regions

Titanium tetraisopropoxide (TTIP) and water were used to selectively deposit titania via ALD in PMMA-free regions. Support that titania was deposited selectively was provided by XPS which showed no detectable Ti 2p in the alumina (previously PMMA coated) region, Figure 4.4 and Table 4.2. The titania-alumina samples for XPS were created in the same way as the ZnO-alumina samples. Samples were treated with 500 TTIP/water ALD cycles and then
PMMA was infiltrated with 300 TMA doses followed by an annealing step for a final thickness of 12 nm and 50 nm of titania and alumina, respectively.

Figure 4.4. XPS results of co-patterned alumina and titania. Spectra are shown for the Al 2p and Ti 2p regions. Red lines are scans from the alumina (previously PMMA coated) region of the sample while black lines are from the titania (PMMA-free) region of the samples. Solid lines are from as-deposited samples, dashed lines are from samples that were etched with 0.01 M nitric acid to remove the thin alumina layer over the titania region. Diagram included at the bottom for clarity.
Table 4.2. XPS results for co-patterned sample with TiO$_2$ and Al$_2$O$_3$ regions before and after a nitric acid etch. Atomic percent rounded to the nearest whole number. < 0.05% indicates the element was below the detection limit of the XPS.

<table>
<thead>
<tr>
<th></th>
<th>TiO$_2$</th>
<th>Al$_2$O$_3$</th>
<th>TiO$_2$ post etch</th>
<th>Al$_2$O$_3$ post etch</th>
</tr>
</thead>
<tbody>
<tr>
<td>O 1s</td>
<td>62 %</td>
<td>61 %</td>
<td>65 %</td>
<td>64 %</td>
</tr>
<tr>
<td>C 1s</td>
<td>12 %</td>
<td>10 %</td>
<td>11 %</td>
<td>7 %</td>
</tr>
<tr>
<td>Ti 2p</td>
<td>10 %</td>
<td>&lt; 0.5%</td>
<td>17 %</td>
<td>&lt; 0.5%</td>
</tr>
<tr>
<td>Al 2p</td>
<td>15 %</td>
<td>28 %</td>
<td>7 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Si 2p</td>
<td>2 %</td>
<td>2 %</td>
<td>&lt; 0.5%</td>
<td>3 %</td>
</tr>
</tbody>
</table>

Previously, selective deposition of titania was accomplished using a PMMA blocking layer to inhibit TTIP/water ALD$^{23}$ as well as titanium methoxide/water ALD.$^{26}$ This led to our selection of TTIP/water as a viable option for the co-patterning process. TiCl$_4$/water does not work for the co-patterning process as TiCl$_4$ will react with the PMMA layer and block TMA diffusion.$^{126}$

4.3.3 Alumina deposits as a monolayer on titania ALD but not ZnO ALD during infiltration

Co-patterning with titania and alumina was a less selective process than co-patterning with ZnO and alumina. As mentioned above, long exposures of TMA to a reactive surface should result in monolayer growth due to the self-limiting nature of ALD precursors. We previously observed this effect when utilizing patterned PMMA on silicon to pattern alumina via infiltration. Following TMA infiltration and anneal, we saw that the thickness of the alumina patterns scaled with PMMA thickness and the number of TMA doses in the PMMA coated regions, while we observed one monolayer of alumina on the SiO$_2$, PMMA-free, region with no dependence on TMA exposure.$^{126}$

In the case of titania-alumina co-patterning we have observed a similar trend. Titania deposits selectively on PMMA-free regions, just like ZnO, but TMA reacts within the PMMA as well
as on top of the titania producing a thin alumina layer, Figure 4.4 (solid lines). Following an etch step for 1.5 hours in 0.01 M nitric acid the thin layer of alumina was partially removed, Figure 4.4 (dashed lines) and Table 4.2. Argon sputtering was able to reduce the aluminum present to 1% following a 15 minute exposure, data not show. The thin film of alumina on titania was expected as part of the infiltration process. As mentioned earlier, we believe this layer of alumina was not present following ZnO-alumina co-patterning because TMA etches ZnO. This is not the case with titania.

With the knowledge that ZnO slowly etches with TMA exposure, it is possible that a thin passivation layer of ZnO could be preemptively deposited after titania ALD to prevent alumina deposition. With process optimization, one could hypothesize that the ZnO thickness could be calculated such that it could be fully removed by the end of the TMA infiltration step. Preliminary studies using a thin sacrificial ZnO layer show promise for increased selectivity. Using only one ALD cycle of ZnO on top of titania, deposited in PMMA-free regions, we were able to decrease the percent of aluminum following the TMA infiltration step from roughly 15% to 7% without the presence of detectable zinc measured via XPS. Optimization of this technique will be the focus of future work.

![Figure 4.5. SEM micrographs from titania-alumina co-patterned samples. Light areas are titania while dark areas are alumina.](image)

A representative SEM micrograph of co-patterned alumina and titania is shown in Figure 4.5. PMMA samples were patterned using electron beam lithography and then treated 500
TTIP/water ALD cycles followed by 300 TMA infiltration doses and then annealed. Light areas in the images represent titania regions while the dark areas represent alumina regions. The three different images highlight co-patterning at various length scales.

4.3.4 Other ALD precursors tested

Several other ALD precursors were examined for selective deposition within the PMMA-free regions. Zirconia was successfully selectively deposited with tetrakis(dimethylamino)zirconium/water at 175 °C. Halfnia was also successfully selectively deposited with tetrakis(dimethylamino)hafnium/water at 160 °C. In both cases TMA infiltration was unhindered and co-patterned samples were formed. However, a thin alumina layer grew on top of the ALD layer as it did with titania, data not shown.

The selective deposition of SiO$_2$ was not successful using tris(dimethylamino)silane/ozone at 160 °C as the ozone etched PMMA. The selective deposition of tungsten was also not successful using WF$_6$/SiH$_4$ at 220 °C as it similarly etched PMMA. The selective deposition of titania via ALD was also attempted using TiCl$_4$/water at 150 °C but TiCl$_4$ interacted with PMMA and blocked TMA diffusion; we have demonstrated that TiCl$_4$ interacts with PMMA previously and could use TiCl$_4$ to infiltrate PMMA for selective deposition.$^{126}$

4.4 Conclusions

Here we have demonstrated a technique that co-patterns two different materials of interest using paired selective deposition. To deposit the first material ALD was used to selectively deposit on silicon using patterned PMMA as a blocking mask. To pattern the second material one ALD precursor was co-reacted with the patterned PMMA itself to selectively deposit within the polymer and then oxidized into the metal oxide of interest upon annealing, Figure 4.1. Fundamentally, this paper represents a proof of concept for a technique that could be used with many more materials. Theoretically this technique could be used to deposit: iridium from Ir(acac)$_3$/O$_2$ (acac = 2,4-pentanedione), platinum from MeCpPtMe$_3$/O$_2$ (Cp = cyclopentadienyl), and ruthenium from RuCp$_2$/air as these materials have been previously selectively deposited using PMMA blocking layers and saw no metal deposition on PMMA
regions. By using one photoresist to pattern two materials the need for complicated alignment steps is avoided.

4.5 Acknowledgements

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CHAPTER 5. Atomic Layer Deposition Coating of Carbon Nanotubes with Zinc Oxide Causes Acute Phase Immune Responses in Human Monocytes In Vitro and in Mice after Pulmonary Exposure

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5.1 Background

The use of carbon nanotubes (CNTs) in industrial and academic settings has increased dramatically in the last decade. CNTs are used in many different areas including electronics, energy storage, sensors, conductive coatings, capacitors, filtration, and drug delivery. Despite these many potential applications, CNTs share geometric similarities with asbestos and thus there is concern for pulmonary fibrosis, a fatal disease characterized by progressive scar tissue accumulation in the lungs. Rodent studies demonstrate that multi-walled CNTs (MWCNTs) or single-walled CNTs (SWCNTs) delivered to the lungs of rats and mice by inhalation, oropharyngeal aspiration or intratracheal instillation cause fibrosis, suggesting a similar health risks to humans. Moreover, MWCNTs or SWCNTs activate pro-fibrotic signaling pathways and stimulate the production of soluble pro-fibrotic mediators by cultured lung cells, including fibroblasts and monocytes/macrophages suggesting that these in vitro cell models are valuable for predicting the inflammatory and fibrotic potential of CNTs in vivo.

Atomic layer deposition (ALD) is a thin-film deposition technique that utilizes self-limiting surface reactions to achieve conformal thin film coatings with precise sub–nanometer thickness control on complex 3D surfaces, including MWCNTs. ALD allows for thin-film surface modification of MWCNTs with a variety of organic, inorganic or hybrid organic-inorganic
molecules, making the applications for these nanomaterials even broader. We previously reported that ALD coating of MWCNTs with aluminum oxide ($\text{Al}_2\text{O}_3$) reduced the ability of MWCNTs to stimulate the production of pro-fibrotic cytokines in cultured human THP-1 monocytic cells in vitro and reduced MWCNT-induced lung fibrosis in mice in vivo. In the present study, we sought to examine the effect of ALD coating of MWCNTs with zinc oxide (ZnO) on the inflammatory and fibrogenic response in human monocytic cells in vitro and after delivery to the lungs of C57BL6 mice in vivo by oropharyngeal aspiration (OPA).

ZnO nanoparticles (ZnO NPs) have numerous applications such as UV protection, bactericidal activity, and incorporation into coatings. ZnO-coated MWCNTs (Z-MWCNTs) are used for a variety of novel applications. For example, aligned Z-MWCNTs form a stable, but reversible, super-hydrophobic material. In addition, ZnO is photocatalytic and Z-MWCNTs can be used as a filter to degrade toxic components of industrial effluents. Z-MWCNTs have also been explored as a nanogenerator. With such a broad range of applications there is significant potential for human exposure to Z-MWCNTs.

The cellular or pathological effects of ZnO-coating on MWCNTs have not been investigated. Herein we report that MWCNTs coated with ZnO by ALD enhanced acute lung inflammation in mice and dramatically increased IL-6 in bronchoalveolar lavage fluid (BALF) and IL-6 mRNA in lung tissue at one day post-exposure. Moreover, IL-6 mRNA levels were increased in heart and liver from mice exposed to Z-MWCNTs, indicating a systemic acute phase immune response. Z-MWCNTs also markedly increased IL-6 mRNA levels in THP-1 monocytes compared to uncoated MWCNTs (U-MWCNTs). Mice exposed to Z-MWCNTs were acutely symptomatic and exhibited lethargy and shivering during the first 24 hours after exposure but regained asymptomatic behavior thereafter. No significant differences in pulmonary fibrosis were observed at 28 days among mice treated with Z-MWCNTs or U-MWCNTs. This study expands the understanding of surface termination on the in vivo pulmonary response by contrasting the increased acute phase response caused by ZnO ALD coating in the present study with decreased toxicity and reduced fibrosis observed previously upon ALD coating of MWCNTs with $\text{Al}_2\text{O}_3$. 

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5.2 Results

5.2.1 ALD on MWCNTs creates a conformal layer of ZnO that modifies physical properties

Atomic layer deposition (ALD) utilizes a set of self-limiting reactions to create thin, conformal coatings, Figure 5.1a. This can be accomplished at a range of temperatures and pressures to achieve the chosen coating. Precursors, in this case diethyl zinc (DEZ) and water, were added one at a time and allowed to react with the surface. Between each step the ALD reactor was purged with nitrogen gas to remove all of the free chemical species. Cycles were repeated A B A B until the desired thickness was achieved. MWCNTs were coated with ZnO using ALD in a mesh basket surrounded by polypropylene to yield ZnO-coated MWCNTs (Z-MWCNTs). This allowed for the conformal coating of a high surface area, fine powder in a traditional ALD reactor. Diffusion through polypropylene and into the bulk MWCNT powder was achieved by allowing the precursors, DEZ and water, to remain in the reactor for a minute before purging. The resulting ZnO thickness was a function of ALD cycles where normal, linear ALD growth of roughly 2 Å/cycle was observed via measurements taken from TEM micrographs, Figure 5.1b. The mass gain per cycle also showed a linear trend with mass increasing along with cycle number.

We observed that the ZnO coating caused several changes in the physical and chemical properties of the MWCNTs. One of the most dramatic physical changes observed in this study caused by ALD coating with ZnO was the increased rigidity of these normally flexible MWCNTs. It was noted from TEM micrographs that the U-MWCNTs were longer than their coated counterparts, Figure 5.1c. The Z-MWCNTs were completely coated with ceramic ZnO after 50 cycles, the cycle number used for these experiments. When these coated tubes were sonicated for 1 min to disperse them in solution, the rigid ceramic coating caused the Z-MWCNTs to break into shorter lengths; yet, very little delamination of the coating was seen and breakage was primarily noted at the tube ends, Figure 5.1c. In contrast, sonication of U-MWCNTs for 1 min did not significantly affect tube length. Quantification of tube length showed that Z-MWCNTs were significantly shorter than U-MWCNTs after sonication, Figure
5.2a. Z-MWCNT breakage upon sonication has also been observed with aluminum oxide coatings.\textsuperscript{93}
Figure 5.1. Atomic layer deposition (ALD) of ZnO on MWCNT. A) Schematic of ALD on a generic surface. Reactants are introduced sequentially to build one self-limiting monolayer of material at a time. B) ZnO thickness and material weight gain increase linearly with the number of ALD cycles. C) TEM micrographs of MWCNT, uncoated and coated, show tube length decreases following sonication. Higher magnification images show conformal coating.

The dispersion of U-MWCNTs (40 µg/mL) was compared to Z-MWCNTs that had been coated with 50 cycles of ZnO as measured by dynamic light scattering in serum free media. A significant decrease in aggregate diameter was seen for Z-MWCNTs as compared to U-MWCNTs, Figure 5.2b. This is likely due to the coating interrupting the Van der Walls interactions that normally occur between U-MWCNTs.\textsuperscript{69,70}
Figure 5.2. Characterization of uncoated MWCNTs (U-MW), ZnO-coated MWCNT (Z-MW), and ZnO nanoparticles (ZnO NP). A) Length of ZnO coated MWCNT decreased post sonication as measured by TEM. B) MWCNT aggregate size decreased with ZnO coating. For the data in panels A and B, The mean is represented as a + and the median as a solid line. For box and whisker plots the box represents the range from the 25th to 75th percentile. Significance is represented as * without a bracket as compared to the control, * denotes P<0.05, ** denotes P<0.01, and *** denotes P<0.001. C) Time course of Zn^{2+} ion concentration generated from Z-MWCNT or ZnO NP in serum free media. Zn^{2+} concentration was measured in serum-free medium in the absence of cells using 200 µg/ml of nanomaterial as described in Methods. Data are the mean and SEM of 5 measurements at each time point. D) H_{2}O_{2} production in serum free media was significantly increased by Z-MWCNT (Z-MW), but not U-MWCNT (U-MW), compared to control. Data are the mean and SEM of 6 replicate measurements. *P<0.05 compared to control.

As ZnO is slightly water-soluble there is potential for harmful concentrations of Zn^{2+} ions to leach from the Z-MWCNTs. The concentration of Zn^{2+} ions in serum free media from Z-MWCNT suspensions (200 µg/mL, all samples) was measured after 24 hour incubation at 37 °C. There were detectable levels of Zn^{2+} in serum free media incubated with Z-MWCNTs or ZnO nanoparticles that increased in a time-dependent manner, Figure 5.2c. Zn^{2+} ions were also measured in supernatants from THP-1 cells cultured for 24 hours with either U-MWCNTs or Z-MWCNTs. A significant increase in Zn^{2+} was measured in the serum-free medium supernatants of THP-1 cells incubated for 24 hours with Z-MWCNTs as compared to an equivalent amount of Z-MWCNTs incubated in serum-free medium in the absence of cells, Figure A.1 (Figures A.1-A-6 are located in Appendix A).

Reactive oxygen species (ROS) were also measured in serum free media containing U-MWCNTs, Z-MWCNTs, or ZnO NPs (200 µg/mL, all samples) since ROS have been implicated as key players in MWCNT-induced pulmonary fibrosis. It has been hypothesized that ZnO interacts with the cell membrane causing damage from electrostatic interaction or direct contact. We measured the hydrogen peroxide (H_{2}O_{2}) concentration after MWCNTs were incubated for 24 hours at 37 °C in serum free media. Z-MWCNTs, but not U-MWCNTs, generated a significant increase in H_{2}O_{2} compared to serum-free medium control, Figure 5.2d.
As a point of comparison, ZnO nanoparticles (NPs) were also tested to determine how the presence of ZnO itself in the absence of MWCNTs would affect the inflammatory response in vitro. ZnO NPs have the smallest diameter and aggregate size of all materials tested, Figure 5.2a,b. ZnO NPs also showed a significant increase in Zn\(^{2+}\) concentration at approximately 1 \(\mu\)M, as compared serum-free medium control, Figure 5.2c. ZnO NPs increased H\(_2\)O\(_2\) above the serum-free media control, albeit not significantly, Figure 5.2d.

5.2.2 Z-MWCNTs stimulate pro-inflammatory cytokine expression by THP-1 cells in vitro

Human THP-1 cells were exposed to U-MWCNTs, Z-MWCNTs or ZnO NPs in vitro to develop predictive information on cytokine expression that could correlate with in vivo responses in mice. All nanomaterials were freshly prepared and sonicated 10 mg/mL stock solutions. Concentrations were as follows: Z-MWCNTs (40 \(\mu\)g/mL), U-MWCNTs (14 \(\mu\)g/mL) and ZnO NPs (26 \(\mu\)g/mL); where the concentration of U-MWCNTs was reduced to 14 \(\mu\)g/mL to normalize for nanoparticle number compared to Z-MWCNTs and ZnO NP concentrations were reduced to 26 \(\mu\)g/mL to match the total ZnO content of the Z-MWCNT samples, see methods section for more details on this calculation. Cell viability determined by Trypan Blue staining demonstrated that the doses of Z-MWCNTs and ZnO NPs produced approximately 25% cytotoxicity while the dose of U-MWCNTs did not cause a significant change in cytotoxicity, Figure A.2. Unprimed, non-adherent THP-1 cells were dosed and then collected 24 hours later via centrifugation. Levels of IL-6, IL-1\(\beta\) and CXCL10 mRNAs were significantly increased for cells exposed to Z-MWCNTs compared to U-MWCNTs and control cells, Figure 5.3a. ZnO NP exposure to THP-1 cells increased mRNA levels of IL-6 and IL-1\(\beta\) as compared to U-MWCNTs and control cells. CXCL10 mRNA was significantly increased with Z-MWCNT exposure but not by U-MWCNTs. TNF-\(\alpha\) mRNA was also elevated for Z-MWCNTs and ZnO NP exposed cells, although not significantly. These trends matched those found in vivo one day after oropharyngeal aspiration with the exception of IL-1\(\beta\), which was increased in THP-1 cells in vitro by Z-MWCNTs but not in the BALF of mice in vivo. Instead, U-MWCNTs increased IL-1\(\beta\) in the BALF of mice yet did not increase IL-1\(\beta\) in THP-1 cells.
*in vitro.* U-MWCNTs were found in the cytoplasm of THP-1 cells 24 hours after treatment, Figure 5.3b. In contrast, Z-MWCNTs were not observed within the cytoplasm of THP-1 cells (data not shown).

Figure 5.3. Cytokine mRNA expression in THP-1 cells 24 hours after exposure to Z-MWCNTs, U-MWCNTs or ZnO NPs. A) Taqman qRT-PCR was used to quantify IL-6, IL-1β, CXCL10 and TNF-α mRNA levels. Significant increases in pro-inflammatory cytokines were observed for both Z-MWCNT and ZnO NP treated cells. Asterisks represent comparisons to the control (*P<0.05, **P<0.01, and ***P<0.001). Asterisks above a bar represent comparison to U-MWCNT. (B) Representative TEM images of THP-1 cells (upper panel) and U-MWCNT within cytoplasm (Cyt) of a THP-1 cell (lower panel). ‘Nu’ denotes nucleus. Arrows indicate U-MWCNTs within THP-1 cells. Z-MWCNTs were not observed within THP-1 cells.
5.2.3 ZnO coating enhances the acute lung inflammatory response to MWCNTs in mice

Mice were exposed to either U-MWCNTs or Z-MWCNTs via oropharyngeal aspiration at 4 mg/kg and 10 mg/kg, respectively, in 0.1% pluronic saline solution so as to dose with the same number of MWCNTs, see methods section for more details on dosing. Control mice were exposed to 0.1% pluronic saline solution alone. Z-MWCNTs were coated with 50 ALD cycles to achieve a thickness of approximately 10 nm. To ensure that the same number of tubes was delivered for U-MWCNTs and Z-MWCNTs, the mass gain from ALD was used to calculate a normalized dose using the linear relationship shown in, Figure 5.1b. As such, Z-MWCNTs were dosed at 2.5 times that of U-MWCNTs since 60% of the mass of Z-MWCNTs was due to the ZnO coating.
Figure 5.4. Cell counts in the BALF of mice exposed to U-MWCNTs or Z-MWCNTs. A) The total cell concentration was significantly increased one day after exposure to Z-MWCNT as compared to control and U-MWCNT exposed mice, B) A significant increase in total cell concentration was also seen at 28 days for U-MWCNTs and Z-MWCNTs. C) After exposure to Z-MWCNTs, macrophages and neutrophils were significantly increased as compared to control. D) Elevated levels of macrophages were seen 28 days after U-MWCNT exposure. Macrophages and neutrophils were still elevated 28 days after Z-MWCNT exposure. The number of animals per group at one day was control (3), U-MWCNT (4), Z-MWCNT (4) and at 28 days was control (4), U-MWCNT (5), Z-MWCNT (3). Significance is represented as * as compared to the control and # as compared to U-MWCNT, * denotes P<0.05, ** or ## denotes P<0.01, and *** denotes P<0.001.

At 24 hours post-exposure half of the mice in the study were euthanized via i.p. pentobarbital overdose followed by collection of BALF from the lungs. Mice exposed to U-MWCNTs showed a 3-fold increase in total number of cells in BALF compared to control at one day post-exposure, Figure 5.4a. Mice exposed to Z-MWCNTs showed a significant and robust increase in total number of cells in BALF at one day post-exposure, a greater than 7.5-fold increase compared to control and a 2 to 3-fold increase above that observed for U-MWCNT treatment, Figure 5.4a,c. By 28 days post-exposure, total cell counts in BALF were reduced compared to one day post-exposure, yet both U-MWCNT and Z-MWCNT exposure groups had significantly elevated total cell counts at 28 days compared to controls, Figure 5.4b. Differential cell counting was performed to identify relative percentages of monocyte/macrophages, neutrophils, eosinophils and lymphocytes in BALF at one and 28 days. The majority of the cells present at one day after U-MWCNT exposure were an approximately equal mixture of macrophages and neutrophils, while cells increased by Z-MWCNTs exposure were represented by twice as many macrophages as compared to neutrophils, Figure 5.4c. The BALF macrophages increased by either U-MWCNTs or Z-MWCNTs were most likely infiltrating monocytes that mature into alveolar macrophages, yet we did not differentiate between monocytes and macrophages when performing BALF cell counts. The majority of cells found 28 days after exposure were macrophages/monocytes for both treatment groups, Figure 5.4d.
Figure 5.5. A) Micrographs of cell populations from BAL fluid showing U-MWCNTs, but not Z-MWCNTs, taken up by macrophages at one day post-exposure. Arrows represent neutrophils. B) Quantification of U-MWCNTs or Z-MWCNTs within macrophages at one and 28 days. One day after nanotube exposure more than 50% of the macrophage population of U-MWCNT-treated mice have visibly engulfed MWCNTs while only about 10% of the macrophages present in the Z-MWCNT treated mice have visibly engulfed MWCNTs. This difference evens out by day 28. The number of animals per group at one day was control (3), U-MWCNT (4), Z-MWCNT (4) and at 28 days was control (4), U-MWCNT (5), Z-MWCNT (3). Significance is represented as ### (P<0.001) between U-MWCNT and Z-MWCNT at one day post-exposure.

5.2.4 ZnO coating prevents MWCNT uptake in the lungs of mice

The relative number of macrophages in BALF that engulfed U-MWCNTs or Z-MWCNTs at one and 28 days was quantified from slides containing BAL cells isolated by Cytospin centrifugation. Interestingly, at one day post-exposure more than half of the macrophages in the BALF of U-MWCNT-treated mice contained MWCNT aggregates that were visible by light microscopy, while only 10% were visible from Z-MWCNT-treated mice. In contrast, by day 28 the number of macrophages visibly containing MWCNTs was approximately equal (30-40%) between U-MWCNT and Z-MWCNT treatments groups, Figure 5.5.

5.2.5 ZnO coating of MWCNTs increases the acute phase lung inflammatory response in mice but does not affect the chronic pulmonary fibrotic response

At day one and 28, lungs from U-MWCNT and Z-MWCNT-exposed mice were fixed and stained with hematoxylin and eosin or Masson’s trichrome stain to evaluate inflammation and fibrosis, respectively. As shown in Figure 5.6, Z-MWCNTs produced a much more robust inflammatory response in the lungs of mice at one day post-exposure compared to U-MWCNT that largely resolved by day 28 in both treatment groups. The increase in inflammation observed by histopathological evaluation at one day post-exposure (Figure 5.6) was consistent with the relative increases in BALF cell differential counts, Figure 5.4. After 28 days, fibrotic lesions at alveolar duct bifurcations in lung sections stained with Masson’s trichrome appeared somewhat larger in mice exposed to Z-MWCNTs compared to mice treated with U-MWCNTs,
Figure A.3. However, quantitation of total lung collagen by Sircol assay showed no significant changes in total lung collagen between treatment groups at either one or 28 days, Figure A.4.

Figure 5.6. Histopathology of hematoxylin and eosin-stained mouse lung sections after exposure to U-MWCNTs or Z-MWCNTs at one and 28 days post-exposure. Treatment with U-MWCNTs caused more focal, condensed lesions at alveolar duct bifurcations (ADB) at day one (arrows) whereas Z-MWCNTs caused a more diffuse inflammatory response in the lower lung around terminal bronchioles (TB), ADB and alveolar ducts (AD). At day 28, both U-MWCNTs and Z-MWCNTs caused focal lesions at ADB (arrows). (*) indicate sites of inflammation.

5.2.6 ZnO coating prevents MWCNT-induced DNA synthesis in airway epithelium of mice

DNA synthesis was measured in the lungs of mice exposed to U-MWCNTs or Z-MWCNTs as an index of lung cell proliferation using bromodeoxyuridine (BrdU) uptake. Airway epithelial cells were the only cells observed to be undergoing DNA synthesis in the lungs of U-MWCNT-exposed mice. BrdU-positive airway epithelial cells were quantified relative to the total
number of airway epithelial cells in photomicrographs from control mice or mice exposed to U-MWCNTs or Z-MWCNTs. Less than 0.5% of total lung cells were observed to uptake BrdU in the control 0.1% pluronic saline group at one or 28 days. One day after exposure to U-MWCNTs, approximately 4% airway epithelial cells were BrdU-positive, indicating cells undergoing DNA synthesis, Figure 5.7. In contrast to mice exposed to U-MWCNTs, no BrdU uptake was observed in the airway epithelium of mice exposed to Z-MWCNTs.

5.2.7  ZnO coating of MWCNTs increases pro-inflammatory cytokines in the lungs of mice

Levels of cytokine proteins (IL-6, IL-1β, CXCL10, and TNF-α) were measured by ELISA in the BALF of mice following exposure to U-MWCNTs or Z-MWCNTs, and the corresponding cytokine mRNA levels were measured by Taqman real time RT-PCR in lung tissue. Z-MWCNTs markedly increased IL-6 protein and mRNA at one day post exposure in the lungs of mice and IL-6 levels returned to control levels by 28 days, Figure 5.8a. In contrast, U-MWCNTs did not induce IL-6 protein or mRNA. IL-1β protein in BALF was increased approximately 2-fold, albeit not significantly, after exposure to U-MWCNTs, but was not increased by exposure to Z-MWCNTs, Figure 5.8b. Z-MWCNTs significantly increased CXCL10 protein and mRNA at one day post-exposure and CXCL10 levels returned to control levels by 28 days, Figure 5.8c. In contrast, U-MWCNTs did not induce CXCL10 protein or mRNA. Neither U-MWCNTs nor Z-MWCNT increased TNF-α protein levels in BALF from mice, yet TNF-α mRNA in lung tissue was significantly elevated by Z-MWCNTs at one day, Figure 5.8d. There were no significant changes in the mRNA levels of the fibrotic mediator OPN in the lungs of mice exposed to U-MWCNT or Z-MWCNT at one or 28 days; mRNA levels of the fibrotic mediator TGF-β1 were significantly decreased by Z-MWCNT treatment compared to control, Figure A.5.
Figure 5.7. Bromodeoxyuridine (BrdU) labeled lung sections highlight airway epithelial cells undergoing DNA synthesis in treated and untreated mice. U-MWCNTs show a significant increase in the number of positively labeled cells as compared to both control and Z-MWCNT treated mice (inset panel shows BrdU positive cells indicated by arrows). The lower right hand panel shows quantitative data expressed as % positive BrdU airway epithelial cells relative to the total number of airway epithelial cells in cross-sectional profiles of small to medium-sized airways in lung sections. The number of animals per group at day one was control (3), U-MWCNT (4), Z-MWCNT (4). Significance is represented as *** (P<0.001) U-MWCNTs compared to Control or Z-MWCNTs at day 1 post-exposure.

5.2.1 Pulmonary exposure to ZnO-coated MWCNTs induces a systemic increase in IL-6 mRNA

Since IL-6 is involved in the systemic acute phase response\textsuperscript{166,167} and because we observed a dramatic induction of IL-6 in lung tissue after Z-MWCNT exposure, we measured mRNA levels of IL-6 in heart, spleen and liver of mice exposed to U-MWCNTs or Z-MWCNTs. IL-6 mRNA was significantly increased in the heart and liver tissue of mice one day after exposure.
to Z-MWCNTs as compared to control mice, Figure 5.9a,c. By day 28, IL-6 mRNA levels in heart and liver returned to control levels. A slight although not significant increase in IL-6 mRNA was also observed one day after treatment in the spleens of mice treated with Z-MWCNTs, Figure 5.9b. Z-MWCNT-treated mice were symptomatic (lethargic and exhibited shivering) within the first 24 hours after exposure, indicative of a systemic acute phase response, but returned to normal behavior by 48 hours. In contrast, U-MWCNT-treated mice were not symptomatic. Of the five mice in the Z-MWCNT 28 day group, one died at day one and one at day six, Figure A.6.

5.3 Discussion

As new applications for MWCNTs appear so do the number and types of functionalized MWCNTs, thus posing new and potentially unanticipated risks for human exposure. Investigations with rodents have already begun to address the biological consequences of MWCNT functionalization. However, few studies evaluate how MWCNT functionalization by ALD surface modification or coating can influence lung disease in experimental animals in vivo. In previous work, we observed a reduced lung fibrotic response to Al₂O₃-coated MWCNTs (A-MWCNTs) compared to uncoated MWCNTs (U-MWCNTs). Moreover, the reduced lung fibrosis observed with A-MWCNTs corresponded to decreased BALF levels of OPN and TNF-α, both which play important roles in inflammation and fibrosis. However, while our previous work showed that A-MWCNTs caused less lung fibrosis than U-MWCNTs in mice at 28 days post-exposure, acute lung inflammation at one day post-exposure was not different between U-MWCNTs and A-MWCNTs. The findings reported here with Z-MWCNTs contrast with previous findings on A-MWCNT toxicity in two ways. First, we show that Z-MWCNTs cause significantly greater inflammation compared to U-MWCNTs in the lungs of mice characterized by the infiltration of monocytes and neutrophils along with high levels of IL-6 and CXCL10. Second, the enhanced lung inflammation observed by Z-MWCNT at one day post-exposure did not result in changes in the amount of fibrosis at 28 days post-exposure. Fibrosis was similar between U-MWCNT and Z-MWCNT treatment groups as determined by histopathology and Sircol collagen assay but
confined to different regions of the lung, likely due to differences in tube aggregation, length and density. Therefore, the findings reported here are novel because they emphasize that the specific chemical composition of surface coatings determine the nature of inflammatory and fibrotic responses caused by ALD-functionalized MWCNTs regardless of where the nanomaterial deposited in the lungs.
Figure 5.8. Lung cytokine protein and mRNA levels at one and 28 days post-exposure to U-MWCNTs or Z-MWCNTs. A) IL-6, B) IL-1β, C) CXCL10 and D) TNF-α protein and mRNA expression. In vivo lung exposure of mice to Z-MWCNTs elevated pro-inflammatory cytokine mRNA and protein levels one day after exposure while exposure to U-MWCNTs did not. Twenty-eight days later all levels are at or below control levels with the exception of TNF-α mRNA. The numbers of animals per group at day one were: Control (3), U-MWCNT (4), Z-MWCNT (4) and at 28 days was: Control (4), U-MWCNT (5), Z-MWCNT (3). Significance is represented as * as compared to the control and # as compared to U-MWCNT, * denotes P<0.05, ** denotes P<0.01, and *** denotes P<0.001.

We observed a significant increase in IL-6 protein and mRNA in the lungs of mice one day after exposure to Z-MWCNTs, as well as elevated IL-6 mRNA levels in the heart and liver indicating a systemic immune response. IL-6 is a pleiotropic acute phase cytokine released in response to inflammatory stimulation and mediates cell proliferation, growth, differentiation, acute phase reactant production in the liver, and fever. Therefore, IL-6 is a likely candidate for mediating the dramatic pro-inflammatory response seen in the lungs of mice treated with Z-MWCNTs as well as the acute fever-like symptoms observed in mice in the first 24 hours after exposure to Z-MWCNTs. CXCL10 was also highly increased in the lungs of mice after exposure to Z-MWCNTs compared to U-MWCNTs. CXCL10 is produced by monocytes in response to interferons secreted by T lymphocytes in response to pathogens in the body. CXCL10 recruits monocytes, macrophages, Th1 lymphocytes, natural killer cells and dendritic cells. Therefore, CXCL10 likely played a role in the high numbers of infiltrating monocytes observed in the BALF and lung tissue of mice treated with Z-MWCNTs at one day post-exposure. CXCL10 also plays an important role in tissue repair and has been shown to have an anti-fibrotic effect in vivo. Therefore, the increased levels of CXCL10 in the lungs of mice exposed to Z-MWCNTs could have played a role in the resolution of the acute inflammatory response observed 1 day after Z-MWCNT exposure.
Figure 5.9. Systemic effects of Z-MWCNT exposure. A) Expression of IL-6 in the heart is elevated significantly one day after lung exposure to Z-MWCNT. B) IL-6 is slightly elevated in the spleen one day after Z-MWCNT exposure. C) Z-MWCNT exposure caused significantly elevated IL-6 in the liver as compared to control and U-MWCNT dosed mice at one day. The number of animals per group at day one was: control (3), U-MWCNT (4), and Z-MWCNT (4) and at 28 days was: control (4), U-MWCNT (5), Z-MWCNT (3). Significance is represented as * as compared to the control and # as compared to U-MWCNT, * denotes P<0.05, ** denotes P<0.01, and *** denotes P<0.001.
Alternative testing using in vitro cell culture models to predict biological responses in vivo has become increasingly important towards evaluating the toxicity of nanomaterials. The human THP-1 monocytic cell line was used in the current study to predict the inflammatory response to Z-MWCNTs in the lungs of mice in vivo. THP-1 cells are increasingly used to study the inflammatory or innate immune responses of macrophages to engineered nanomaterials. THP-1 cells are an appropriate cell culture model since circulating monocytes differentiate into macrophages after they infiltrate into lung tissue in response to an inflammatory stimulus and macrophages represent a first line of defense in the lungs by engulfing MWCNTs. In the present study, IL-6 and CXCL10 mRNA expression in THP-1 cells in vitro induced by Z-MWCNTs closely matched the same pattern of induction for these two cytokines by Z-MWCNTs in the lungs of mice in vivo. TNF-α mRNA was induced in vivo in the lungs of mice by Z-MWCNTs only at the mRNA level, and while this same trend in TNF-α mRNA induction was observed in THP-1 cells in vitro, it was not statistically significant. However, Z-MWCNTs also increased IL-1β mRNA levels in THP-1 cells in vitro, but IL-1β was not induced by Z-MWCNTs in vivo. IL-1β is a cytokine released from macrophages and is a mediator of inflammation. Therefore, our in vitro cytokine expression was only partly predictive of in vivo cytokine expression. The in vitro responses of THP-1 cells to MWCNTs can be modified by a variety of factors, including LPS priming and/or treatment with phorbol ester to differentiate monocytes to macrophages. In the present study, THP-1 cells were neither primed with LPS nor stimulated with phorbol ester and thus represented a monocyte phenotype.

Interestingly, U-MWCNT stimulated airway epithelial DNA synthesis in the airway epithelium of mice as measured by BrdU uptake, whereas no significant DNA synthesis was observed in the airway epithelium of Z-MWCNT-exposed mice. The airway epithelial proliferative response one day after U-MWCNT exposure is similar to the response observed in rats after inhalation exposure to chrysotile asbestos. The incorporation of BrdU into airway epithelial cells following U-MWCNT exposure likely represents a response to injury where DNA synthesis and cell cycle progression are initiated to allow for epithelial cell
proliferation as part of a homeostatic repair process. The lack of BrdU incorporation in airway epithelium after exposure to Z-MWCNT suggests that the ZnO coating or dissolution of Zn\(^{+2}\) ions causes epithelial cell growth arrest. Cell cycle arrest has been reported in RSC96 Schwann cells and in epidermoid cancer cells exposed to varying concentrations of ZnO NPs \textit{in vitro}.\(^{178,179}\) Both of these previous studies cited the ability for ZnO NPs to increase ROS and thus induce DNA damage thereby halting DNA synthesis. H\(_2\)O\(_2\) has recently been demonstrated as a primary mediator of Zn-induced oxidative stress in human airway epithelial cells.\(^{180}\) The present study shows that Z-MWCNTs increased H\(_2\)O\(_2\) in cell-free media. In addition, we detected Zn\(^{+2}\) ions after incubation of Z-MWCNTs in media, indicating some degree of dissolution. Therefore, it is possible that growth arrest of the airway epithelium in mice exposed to Z-MWCNTs is due a ROS-dependent mechanism involving dissolution of Zn\(^{+2}\) ions. Alternatively, ROS could be generated from the surface of Z-MWCNTs.

Previous studies have shown that ZnO causes toxicity and lung injury through the release of Zn\(^{+2}\) or via ROS-dependent mechanisms. For example, the soluble fraction of combustion emission particulate matter mediates lung inflammation in rats and this is due in part to dissolution of metal ions, including Zn\(^{+2}\).\(^{181}\) In addition, occupational inhalation exposure of welders to Zn causes an acute lung inflammatory response referred to as “metal fume fever” and this is largely mediated via soluble Zn\(^{+2}\).\(^{182}\) Moreover, it has been shown that ZnO can generate ROS, specifically H\(_2\)O\(_2\), via an aqueous phase reaction with oxygen vacancies within the ZnO crystal lattice.\(^{183}\) Some evidence suggests that ROS do not have a large contribution to ZnO toxicity, citing that the antioxidant NAC commonly used in studies of ZnO-induced ROS generation is a chelator of zinc and is therefore only reducing cytotoxicity due to the sequestration of zinc ions in solution.\(^{84}\)

It is also a point of controversy as to where the ZnO is dissolving; inside or outside of cells. Xia and coworkers found that non-dissolved ZnO NPs were taken up by BEAS-2B epithelial cells in caveolae by fluorescently labeling the nanoparticles and staining for calveolin-1.\(^{85}\) This same study showed that uptake of ZnO NP by RAW 264.7 macrophages occurred in lysosomes that completely dissolved the nanoparticles.\(^{85}\) Further work by these authors concluded that
although there is some dissolution of ZnO in the media, the main contributor to dissolution is ZnO NP uptake and dissolution within the cell. This is contrasted by a report from Buerki-Thurnherr and colleagues, wherein they concluded that dissolution primarily occurs in the media using Jurkat cells as they were unable to visualize any nanoparticles in the cells via TEM. We have found that ZnO dissolves slowly in serum-free defined media (SFDM) with a slow increase in the concentration of Zn$^{2+}$ seen between one and 48 hours, Figure 5.2c. Additionally, we have observed that when dissolution in SFDM alone was compared to dissolution in SFDM with THP-1 cells present the samples with cells had concentrations almost 7 times higher than the samples without cells, Figure A.1. However, as discussed below, Z-MWCNTs were not avidly taken up by phagocytes in vitro or in vivo. Therefore, enhanced Zn$^{2+}$ dissolution from Z-MWCNTs in the presence of cells apparently does not require cellular uptake.

Finally, our data show that Z-MWCNTs evaded macrophage uptake in the lungs of mice at one day post-exposure and in THP-1 cells in vitro, whereas U-MWCNTs were avidly engulfed by macrophages in vivo and by THP-1 cells in vitro. The reason for evasion of macrophage uptake by Z-MWCNTs remains unclear and requires further investigation. However, Z-MWCNTs not taken up by macrophages would have a greater opportunity to interact with the lung epithelium and cause toxicity. Collectively, our data suggest that the ZnO coating on Z-MWCNTs causes airway epithelial growth arrest through the release of H$_2$O$_2$ and this could be due either to release of Zn$^{2+}$ ions through dissolution or through direct interaction of the surface of Z-MWCNTs with epithelial cell membranes at the nano-bio interface.
Figure 5.10. Illustration of proposed mechanisms underlying the acute phase immune response to ZnO-coated MWCNTs (Z-MWCNTs) in human THP-1 monocytes in vitro and after delivery to the lungs of mice in vivo by oropharyngeal aspiration. Uncoated MWCNTs (U-MWCNTs) are subjected to atomic layer deposition (ALD) coating with ZnO to yield Z-MWCNTs. Sonication results in breakage of Z-MWCNTs and the ZnO coating also undergoes partial dissolution to release Zn$^{2+}$ ions in aqueous media. Unlike U-MWCNTs, Z-MWCNTs are not taken up by phagocytic monocytes or macrophages in vitro or in vivo. Z-MWCNTs cause epithelial cell growth arrest and increases in CXCL10, a monocyte chemoattractant, as well as increases in IL-6 that mediates systemic acute phase responses.

In addition to changing the surface chemistry, the ZnO coating also changes the length and aggregation of the Z-MWCNTs. ZnO is a brittle ceramic material. Once coated by ALD, Z-MWCNTs break into smaller, more dispersed segments after sonication. Agglomeration of MWCNTs is due to Van der Waals interactions, making them difficult to disperse.\textsuperscript{69,70} By coating the tubes there is the potential to interrupt these interactions. Previous studies have suggested that both MWCNT dispersal state and length play a role in inducing fibrosis. Agglomerated MWCNTs produce granulomas in the lungs of rodents, while dispersed...
MWCNTs lead to diffuse interstitial pulmonary fibrosis. MWCNT length also influences cellular response and toxicity. For example, long, rigid materials lead to frustrated phagocytosis by macrophages, resulting in lysosomal membrane damage and release of ROS and pro-inflammatory cytokines. Moreover, decreasing fiber or tube length (i.e., aspect ratio) results in decreased toxicity and more rapid clearance from lung tissue. In the present study, we found that shorter and better dispersed Z-MWCNTs caused more acute inflammation than U-MWCNTs. While the acute phase immune response to Z-MWCNTs observed in this study was likely mediated by surface ZnO and generation of H$_2$O$_2$, greater dispersal of nanotubes could also play a role by increasing bioavailability in the lungs of exposed animals.

In a broader context, the present study extends a growing literature showing that functionalization of MWCNTs can alter biological responses and thereby potentially pose unanticipated hazards for human exposure. However, unlike the enhanced toxicity and immunogenic reactions seen with Z-MWCNTs compared to U-MWCNTs in the present investigation, other studies have shown that certain types of functionalization can decrease the toxic response to MWCNTs. For example, COOH-functionalized MWCNTs induced less lung inflammation and reduced fibrosis in the lungs of mice compared to pristine MWCNTs. As mentioned previously, Al$_2$O$_3$ coating of MWCNTs applied by ALD reduced lung fibrogenesis. Therefore, with regards to ALD functionalization, the chemical composition of the thin-film coating determines lung toxicity and pathologic outcome. Since novel applications of ZnO-coated MWCNTs are increasing in diversity, our work in the present study has important human health implications for exposure to these functionalized nanomaterials.

5.4 Conclusions

In summary, we report that functionalization of MWCNTs by atomic layer deposition (ALD) with ZnO increased pro-inflammatory cytokine expression by THP-1 monocytic cells in vitro and caused an acute phase lung and systemic immune response in mice one day after exposure.
by oropharyngeal aspiration. Interestingly, pulmonary fibrosis induced by Z-MWCNT at 28 days post-exposure was not significantly different from that seen with U-MWCNTs. As illustrated in Figure 5.10, the mechanism underlying the acute phase inflammatory response to Z-MWCNTs involves dissolution of Zn$^{+2}$ ions from the ZnO coating, H$_2$O$_2$ generation, growth arrest of airway epithelial cells, and induction of CXCL10 and IL-6. These observations contrast with previous findings of reduced lung toxicity and fibrosis by MWCNTs functionalized by ALD-coating with Al$_2$O$_3$ and emphasize the importance of chemical composition as a primary factor of the pulmonary innate immune response to ALD-functionalized MWCNTs.

### 5.5 Methods

**Chemicals and Materials.** Diethylzinc (DEZ) (Strem Chemicals, min 98% pure) was used as received. DEZ was co-reacted with deionized (DI) water. The reactor was purged with high purity nitrogen gas (Machine & Welding Supply Co) that was further purified with a Entegris GateKeeper located directly upstream from the reactor input. Silicon substrates (University Wafers, P-type, <100>) were used to monitor the growth of zinc oxide. Multi-walled carbon nanotubes (MWCNT) (Helix Materials Solutions, 0.5-40 um in length) were coated as received. Zinc oxide nanoparticles (ZnO NP) (UC CEIN) were used as a positive control as received.

**MWCNT Atomic Layer Deposition (ALD).** MWCNTs were coated utilizing a method previously described.$^{164}$ Briefly, approximately 30 mg of MWCNTs were placed into a mesh cylinder surrounded by a nonwoven polypropylene (PP) sheet (melt-blown, NC State University, College of Textiles) and secured using white, 100% cotton thread. The PP sheet was measured so as to minimize material overlap and promote diffusion of atomic layer deposition (ALD) precursors. A silicon wafer monitor was placed upstream of the encased MWCNTs and similarly wrapped. Behind the MWCNTs was placed an unwrapped silicon wafer monitor. Samples were placed into a custom made, viscous-flow, hot-walled, vacuum reactor.$^{2,155,187}$ The reactor was kept at roughly 800 mTorr, and operated at 35 °C. DEZ was
introduced into the reactor and held by closing all ports into and out of the reactor for 60 seconds; this allowed for proper diffusion of the precursor through the PP. The reactor was then purged with N₂ gas. This was followed by a co-reacting step with DI water that was also allowed to be held in the reactor for 60 seconds.

**MWCNT Characterization.** ZnO coating thickness was determined by spectroscopic ellipsometry (J.A. Woollam Co., Inc) of the monitor silicon wafers. Thickness was also measured using a JEOL 2000FX scanning transmission electron microscope (TEM). TEM samples were prepared by dropping 3 µl of ENM (engineered nanomaterials) suspended in 100% ethanol on to a carbon faced TEM grid (Protochips) and allowing the suspension to dry. Samples were sonicated using the method described above before TEM grid preparation. From TEM images the length of the ENM was measured. ImageJ software was used to measure MWCNT length and ZnO thickness.

Mass gain of the MWCNT after the ALD coating process was measured (Fischer Scientific, accuSeries-accu124). This allowed for validation that the nanomaterial was being coated, and also for the potential to correct for this weight change when dosing to normalize to the number of MWCNT dosed instead of the total weight.

Dynamic light scattering (DLS, Malvern Zetasizer ZSP) was used to determine the MWCNT aggregate size. ENM were suspended as described above and then diluted to a concentration of 40 µg/mL in serum free media, the same that was used to serum starve cells. Following one hour of settling, shorter times resulted in inconsistent data as large aggregates actively settled, the samples were measured. Values were reported using the number percent of the diameter measured. Three different samples were used to establish significance.

Zn^{2+} concentrations were measured with a NanoMolar Zinc Assay Kit (ProFoldin) according to manufacturer’s instructions. ENM were incubated in serum free media for 24 hours in the dark at 37 °C at a concentration of 200 µg/mL. Florescence was read using a FLUOstar Omega (BMG Labtech).
H$_2$O$_2$ concentrations were measured using an Amplex Red Assay (Thermo Fisher Scientific) according to the manufacturer’s instructions. U- and Z-MWCNTs or ZnO nanoparticles were incubated in serum free media for 24 hours in the dark at 37 °C at a concentration of 200 μg/mL. Absorbance was read at 560 nm using a microplate spectrophotometer (Multiskan EX, ThermoFisher Scientific).

*Cell Culture and Dosing.* Human monocytes (THP-1) (ATCC) were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Gibco) and kept at 37 °C with 5% CO$_2$. Cells were transferred into 35 mm plates (Becton Dickinson) for experimentation at a concentration of approximately 4.5x10$^5$ cells/mL. Once plated, cells were serum starved in F-12K Nutrient Mixture with albumin solution and ITS (Gibco, Sigma- 35% in DPBS, and Lonza respectively) overnight. The concentration range of MWCNT used dosing THP-1 cells in vitro was consistent with that previously established through intra-laboratory consortium testing of engineered nanomaterials. Cells were dosed with a stock solution (10 mg/mL) of engineered nanomaterials (ENM, in this case MWCNT that were either uncoated or coated in ZnO as well as ZnO NP) in sterile 0.1% pluronic F-68 (Sigma-Aldrich). The dose of U-MWCNTs, Z-MWCNT and ZnO NP were 14, 40 and 26 μg/mL, respectively. The Z-MWCNT dose was normalized to achieve a dose with as similar a number of nanoparticles as possible to the uncoated tubes. To adjust this the mass gain following ALD was utilized in equation 1:

$$MWCNT \text{ dose} \times \frac{m_{\text{coated MWCNT}}}{m_{\text{uncoated MWCNT}}} = Z-MWCNT \text{ dose} \quad \text{Eqn. 1}$$

Where “m” is the mass of the nanomaterial in grams and the dose is in μg/mL. In this case the value for the mass of coated MWCNT divided by the mass of uncoated MWCNT for this experiment was 2.8 and thus the 14 μg/mL U-MWCNT dose corresponds to a 40 μg/mL Z-MWCNT dose. The ZnO NP dose was such that the total mass of ZnO in the Z-MWCNT dose and the ZnO NP dose was equivalent. To calculate this the following equation was used:

$$ZnO \text{ NP dose} = Z-MWCNT \text{ dose} \times \frac{m_{\text{coated MWCNT}} - m_{\text{uncoated MWCNT}}}{m_{\text{coated MWCNT}}} \quad \text{Eqn. 2}$$
In this case the ratio between the difference of the coated and uncoated tubes to the coated tubes was 0.65 corresponding to 65% of the coated tubes being comprised of ZnO. Vials of ENM were suspended using a cuphorn sonicator (Qsonica) at room temperature immediately preceding dosing using 7 amps, 50 W for a total energy of 2910 J on average.

Cell Viability. Cell viability was determined using a 0.4% Trypan Blue solution (Life Technologies) according to the manufacturer’s protocol. Briefly, Trypan Blue was mixed 1:1 with the THP-1 cell suspension and the number of living and dead cells was counted using a hemocytometer.

Mouse exposure to MWCNTs. All animal procedures were approved by the NC State University Institutional Animal Care and Use Committee (Protocol #13-086-B). C57BL6 mice (Jackson Laboratories) were exposed to U-MWCNTs or Z-MWCNTs (50 ALD cycles) suspended in 0.1% pluronic saline solution via oropharyngeal aspiration under isoflurane anesthesia at 4 mg/kg and 10 mg/kg, respectively, in order to deliver the same number of MWCNTs per mouse. The control group of mice was exposed to pluronic alone. Each treatment group (Control, U-MWCNT, Z-MWCNT) contained 3, 4 and 4 animals at one day, respectively, and 4, 5, and 5 animals at 28 days, respectively. To ensure that the same number of tubes was delivered for U-MWCNTs and Z-MWCNTs, the mass gain from ALD was used to calculate a normalized dose. To do this calculation the same approach was used as in the above section. In this case the batch of Z-MWCNT used had a value of 2.5 for the ratio of mass of coated MWCNT/mass of uncoated MWCNT. As such, Z-MWCNTs were dosed at 2.5 times that of U-MWCNTs for a total of 10 mg/kg. At day one and 28 after exposure, mice were euthanized via intraperitoneal injection of pentobarbitol (Fatal Plus, Vortech Pharmaceuticals). Bronchoalveolar lavage fluid (BALF) was collected from the lungs via two serial lavages of 0.5 mL of phosphate buffered saline (PBS, Dulbecco) and combined. BALF was used to determine cells/mL, cell type and protein content via enzyme-linked immunosorbent assay (ELISA). The caudal and middle lobes of the right lung were stored in RNAlater (Ambion) and used to determine mRNA profiles (as well as heart, liver and spleen). The left lungs were fixed for 24 hours using 10% neutral buffered formalin via intratracheal infusion and then
transferred to 70% ethanol. The left lung was then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) or Masson’s trichrome and imaged using an Olympus BX40 light microscope.

**Bromodeoxyuridine (BrdU) Immunohistochemistry.** For BrdU incorporation, each animal received an intraperitoneal injection of 100mg/kg from a stock of 10mg/mL BrdU (Sigma #B5002) in PBS 1 hr prior to sacrifice. Paraffin blocks were cut 5 μm with a microtome and mounted on a negatively charged slide and dried overnight. The sections were then hydrated and immunostained with anti-BrdU Pure (BD#347580) followed by the vectastain ABC kit (VectorLabs#PK-6102) and DAB buffer (BioGenex#HK542-XAK) as described per manufacturer inserts. The positive brown cells uniquely stood out from the hematoxal counterstain. Quantification of BrdU positive cells was achieved by counting the number of BrdU positive cells as well as the total number of cells per airway. All of the airways from each mouse for each treatment were combined and data was reported as a percentage of BrdU positive cells per treatment group.

**Sircol Collagen Assay.** Soluble collagen in lung tissue was measured by Sircol assay (Biocolor, Carrickfergus, UK) according to the manufacturer’s instructions.

**Cytology.** Cell concentrations from the BALF were determined via hemocytometer. A Cytospin 4 (Thermo-Fisher Scientific) was used to deposit cells from the BALF onto glass slides. Cells were then fixed and stained using a Diff-Quik Stain Set (Siemens). Relative percentages of macrophages, neutrophils, eosinophils, or lymphocytes per 500 cells were then identified using a light microscope. The percent of macrophages visually containing U-MWCNTs or Z-MWCNTs per 100 cells per mouse was also determined.

**RT-PCR.** SuperScript™ III Platinum One-Step qRT-PCR system (Life Technologies) was used in conjunction with a StepOnePlus Real-Time PCR System (Applied Biosystems) to determine the fold change of mRNA for IL-6, IL-1β, CXCL10, TNF-α, OPN, and TGF-β1. RNA was extracted from homogenized lung, heart, liver and spleen tissues using Quick-RNA™ MiniPrep (Zymo Research) according to the manufacturer’s instructions. 18S was used as an
endogenous control “housekeeping gene” for all in vitro experiments. THP-1 cells were collected from suspension via centrifugation for 5 minutes at 1000 rpm. B2M was used as the endogenous control for all mouse experiments.

ELISA. IL-6, IL-1β, CXCL10, TNF-α, OPN, and TGF-β1 protein levels in the BALF were measured via ELISA (DuoSet, R&D Systems). Samples were assayed according to manufacturer instructions. Absorbance was read at 450 nm by a microplate spectrophotometer (Multiskan EX, ThermoFisher Scientific) with a correction wavelength of 540 nm.

Data and Statistical Analysis. Data and statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc.). A one-way ANOVA with a post hoc Tukey test was used to determine significance between samples. A significance of p<0.05 was used unless otherwise noted. Data values were expressed as mean ± SEM.

5.6 Acknowledgements

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CHAPTER 6. Aluminum doping of ZnO Coatings on Multi-Walled Carbon Nanotubes
Alters the Coating Dissolution as well as Cell Type Dependent Cytotoxicity and
Cytokine Production

Erinn C. Dandley, Shannon Diachina, Gregory N. Parsons, James Bonner

This section is an article in preparation and may be revised for future publication.

6.1 Common Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALD</td>
<td>atomic layer deposition</td>
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<tr>
<td>AZO</td>
<td>aluminum doped ZnO</td>
</tr>
<tr>
<td>U-MWCNT</td>
<td>uncoated multi-walled carbon nanotubes</td>
</tr>
<tr>
<td>MWCNT(s)</td>
<td>shortened MWCNT</td>
</tr>
<tr>
<td>A-MWCNT</td>
<td>MWCNT with ~10 nm of Al₂O₃</td>
</tr>
<tr>
<td>Z-MWCNT</td>
<td>MWCNT with ~10 nm of ZnO</td>
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<tr>
<td>AZO-MWCNT</td>
<td>MWCNT with ~10 nm of AZO</td>
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<tr>
<td>ENM</td>
<td>engineered nanomaterial</td>
</tr>
<tr>
<td>SFDM</td>
<td>serum-free defined media</td>
</tr>
<tr>
<td>HLF-16Lu</td>
<td>human lung fibroblasts</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>human bronchial epithelial cells</td>
</tr>
<tr>
<td>THP-1</td>
<td>human monocyte</td>
</tr>
<tr>
<td>THP-1+LPS</td>
<td>LPS primed monocyte</td>
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<tr>
<td>THP-1+LPS+PMA</td>
<td>LPS primed monocyte derived macrophage</td>
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6.2 Background

The increasing use of engineered nanoparticles (NP) in a broad range of applications has raised concerns for human exposure and possible adverse toxicological outcomes. For example, zinc oxide (ZnO) NPs are used for cell imaging, drug delivery, targeted cancer treatment, solar cells, sensors, photo catalyst, pigments, cosmetics, sunscreen, antibacterial ointments, lotion, mouthwash, UV light emitting diodes, laser diodes, and optoelectronic devices. However, numerous studies show that exposure to nano-scale ZnO causes an inflammatory response and or toxicity in rodents.

In addition to spherical NPs other studies have focused on the toxicological effects of multi or single-walled carbon nanotubes (MWCNT, SWCNT) in vitro using cell lines or in vivo using rodent models. Concern over the toxicity of this high aspect ratio nanomaterial is due to its physical similarities to asbestos and to its utility in many different areas including: electronics, energy storage, sensors, drug delivery; organic light emitting diodes, transparent conductive coatings, capacitors, filtration; high strength materials, electronics; contrast imaging agent and drug delivery.

In a new wave of application driven research, MWCNT are being coated with various materials to improve or alter their physical and chemical characteristics thus opening up a new breed of functionalized nanomaterials. Due to ZnO’s many unique properties, ZnO coated MWCNT are being studied as piezoelectric generators, photocatalytic filters, and super-hydrophobic materials. Aluminum (Al) doping of ZnO increases the conductivity of ZnO with the highest conductivity resulting from 3-7% Al. Aluminum doped ZnO (AZO) is the best candidate to replace indium tin oxide (ITO) as the premier transparent conductive oxide (TCO) for transparent devices such as solar cells, flat panel displays and light emitting diodes. AZO-CNT nanocomposites are currently being studied as part of flexible thin film transistors and ITO replacements in TCO devices.
Research has found that high aspect ratio materials are often more toxic than their spherical counterparts. For example, titanium dioxide, which is generally accepted as fairly benign in the form of a nano-sphere, produced increased cytotoxicity and inflammation when in the form of a nano-belt with high aspect ratio.\textsuperscript{193} Similarly, spherical carbon black nanoparticles are relatively benign as compared to carbon nanotubes with respect to pro-inflammatory and profibrogenic potential.\textsuperscript{162} Altering surface chemistry of high aspect ratio nanomaterials could either increase or decrease toxicity. This has lead us to question the safety of surface modified MWCNT.

Our group previously reported that mice exposed via oropharyngeal aspiration to MWCNT coated with alumina (A-MWCNT) by atomic layer deposition (ALD) displayed decreased levels of fibrosis one month after exposure as compared to mice exposed to uncoated MWCNT (U-MWCNT).\textsuperscript{93} Recently we have determined that ZnO coatings on MWCNT (Z-MWCNT) elicits an acute inflammatory response in mice as compared to U-MWCNT.\textsuperscript{158} These studies collectively show that different types of metal oxide coatings applied by ALD have widely different toxicological effects in the lungs of mice. The toxicological consequences of ALD coating MWCNT with different metal oxides in sequence remains unknown. Here we focus on the comparison of four different high aspect ratio materials: U-MWCNT, A-MWCNT, Z-MWCNT and AZO-MWCNT.

It is generally accepted that the toxicity of ZnO is at least partially tied to Zn ion (Zn$^{2+}$) release but the extent of which is unknown.\textsuperscript{73} Some believe that ZnO toxicity is dependent on ROS formation, lysosomal and mitochondrial damage from altered Zn$^{2+}$ homeostasis and enzyme activity inhibition.\textsuperscript{73} Differences have been observed between ZnO NP and Zn$^{2+}$ after epithelial cell exposure and found ions alone to be less toxic.\textsuperscript{83} Cho et al. found a similar response between lung exposure to NP and ions in rats but did see an increase in rat death following the highest dose of ions but not NP.\textsuperscript{75} Other studies have also concluded that Zn$^{2+}$ play a role in ZnO toxicity but saw differences between ions and NP.\textsuperscript{75,81,82}
Each of these studies used one or two different cell lines either with or without an in vivo rodent model. Arguments have been made for dissolution before cellular uptake and after cellular uptake. We believe that through the study of three different cell lines (human fibroblasts, epithelial cells, and monocytes the latter of which was primed in three different ways) at one time we can shed light on the contribution of \( \text{Zn}^{2+} \) to toxicity. We have found that some cell types increase the amount of \( \text{Zn}^{2+} \) in solution while others decrease the concentration of \( \text{Zn}^{2+} \) in solution as compared to media without cells. This has led us to postulate that neither ion dissolution within an aqueous environment nor particle uptake alone is responsible for ion release.

In addition to three different cell lines we have included four different MWCNT: U-MWCNT, A-MWCNT, Z-MWCNT, and AZO-MWCNT. U-MWCNT, A-MWCNT and Z-MWCNT all have in vivo benchmarks previously published by our group.\(^{93,158}\) AZO-MWCNT was included for its industrial relevance and its similarity to the two other coatings studied.

Aluminum doping of ZnO alters the crystallinity of ZnO;\(^{91}\) with a high enough concentration of aluminum films become amorphous and with just a small amount films exhibit decreased surface roughness and crystallinity.\(^{87}\) AZO has decreased solubility as compared to ZnO.\(^{72,90}\) Less research has been done to explore the toxicity of AZO NP. The few studies that have been done contradict one another with some claiming AZO NP to be less toxic than ZnO NP and vice versa.\(^{72,90,92}\) Our main interest was to determine the relative toxicity of AZO coatings on MWCNT relative to A- or Z- coatings alone applied by ALD and to determine if the decreased concentration of \( \text{Zn}^{+2} \) is a good indicator that contributes to toxicity.

The large variations in \( \text{Zn}^{2+} \), cytotoxicity and IL-1\( \beta \) release seen between cell types has shed light on why the contribution of \( \text{Zn}^{2+} \) from ZnO NP has been so hotly debated. We have found that in the THP-1 cell line there were differences in ion concentrations between Z-MWCNT and AZO-MWCNT treatments. Following THP-1 exposure differences were also observed between treatments with Z-MWCNT and AZO-MWCNT with respect to cytotoxicity and IL-1\( \beta \) release. This suggests that \( \text{Zn}^{2+} \) plays a role in toxicity in vitro but the method of dissolution
is different between cell types as was observed as an order of magnitude difference in ion concentrations.

6.3 Results

Three different cell lines were used to determine the biological effects of atomic layer deposition (ALD) coatings on multi-walled carbon nanotubes (MWCNT). Cell types used include human lung fibroblasts (HLF-16Lu), human lung epithelial cells (BEAS-2B) and human monocytes (THP-1). The latter of which was differentiated in three ways: THP-1 cells were either cultured as received (THP-1, monocytes), supplemented with LPS at the time of seeding (THP-1+LPS, activated monocytes), or primed with PMA one hour prior to seeding and also supplemented with LPS at the time of seeding (THP-1+LPS+PMA, monocyte derived macrophages) to differentiate them from a monocyte to macrophage phenotype. MWCNTs with three different ALD coatings were used: alumina (A-MWCNT), ZnO (Z-MWCNT) and aluminum doped ZnO (AZO-MWCNT) as well as shortened MWCNT (MWCNT(s)) to determine the contribution of tube length.

6.3.1 MWCNT Characterization

The length, coating thickness, zeta potential, aggregate diameter and sample crystallinity are represented in Figure 6.1. MWCNT length was determined from measurements taken from TEM images, Figure 6.1a. During the sonication step used to disperse the engineered nanomaterials (ENM, coated and uncoated MWCNT) tubes with coatings were shortened. The coatings are brittle, unlike the U-MWCNT, and cause the tubes to break more easily. For some experiments the effect of tube length was examined. For these experiments both as received MWCNT and MWCNT shortened to the length of the coated MWCNT were utilized.
Figure 6.1. MWCNT characterization. a,b) Using a TEM 50-200 ENM were examined per condition for tube length and coating thickness. c,d) The zeta potential and aggregate diameters were determined for all MWCNTs at 40 µg/mL by weight in SFDM, three samples per condition were each measured three times. e) Schematic representation of the AZO coated MWCNT. f) XRD measurement of Z-MWCNT and AZO-MWCNT to show sample crystal structure. For box and whisker plots the box represents the range from the 25th to 75th percentile, the mean is represented as a + and the median as a solid line. Significance is represented as # as compared to U-MWCNT and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

The atomic layer deposition (ALD) coating thickness was also determined from TEM images, Figure 6.1b. All coatings were targeted to be roughly 10 nm thick. Due to differences in growth rates between ALD processes this meant 82 alumina cycles, 50 ZnO cycles and 5/53...
alumina/ZnO cycles for A-MWCNT, Z-MWCNT and AZO-MWCNT, respectively. Further
detail into the doping of AZO-MWCNT can be seen in Figure 6.1e. First one cycle of ALD
alumina was applied to the sample. This was followed by 13 cycles of ALD ZnO and then 1
cycle of alumina. This was repeated four times and capped with a final ZnO layer. The ratio of
1:13 was chosen for its favorable physical and chemical properties.87

Figure 6.2. Representative TEM images of each of the ENM. ALD coating-MWCNT
boundaries indicated with white bars.

The zeta potential of each of the ENM were measured in serum free media (SFDM) at 40
μg/mL after allowing one hour for settling, Figure 6.1c. U-MWCNT had the largest range of
zeta potential measured as well as the lowest potential. Shortening the MWCNT tightened the
distribution while also slightly increasing the zeta potential for MWCNT(s). A-MWCNT had
the least negative zeta potential followed by Z-MWCNT and AZO-MWCNT.
The aggregate diameter in SFDM for each ENM was also examined after one hour of settling, Figure 6.1d. MWCNT(s), A-MWCNT and AZO-MWCNT all had significantly larger aggregate diameters than U-MWCNT while Z-MWCNT had the smallest aggregate size.

Alumina doping of ZnO has been known to alter the crystal structure of ZnO. To examine this we used x-ray diffraction on Z-MWCNT and AZO-MWCNT, Figure 6.1f. Characteristic peaks of MWCNT and ZnO are indicated on the graph. No change was observed in the intensity of the MWCNT peak. All of the ZnO peaks were observed to decrease upon aluminum doping. A decrease in peak intensity is associated with a decrease in crystallinity.

Representative TEM images of each ENM are shown in Figure 6.2. Alumina deposits as an amorphous layer on the MWCNT. ZnO deposits in a polycrystalline layer on top of the MWCNT. By doping with alumina we can see that the film becomes more conformal with less noticeable crystal units. All three coatings were present on the MWCNT despite sonication to disperse the samples prior to TEM grid preparation.

6.3.2 ALD coating dissolution and pH dependence

To determine the release of Zn into solution with and without aluminum doping a time course experiment was conducted, Figure 6.3a. Z-MWCNT and AZO-MWCNT were incubated in SFDM for 1-48 hours at 37°C at a concentration of 200 µg/mL normalized to the number of Z-MWCNT particles, see methods section for details. Z-MWCNT samples consistently released more ions than the AZO-MWCNT samples. The concentration of ions continued to increase for the 48 hours tested. The concentration of ions in solution in SFDM alone was below the detection limit of the assay used.

The release of ions into solution by ZnO is pH dependent. Knowing this we wanted to test the pH of SFDM from culture with our different cell types without any nanomaterial present, Figure 6.3b. Supernatant was tested 24 hours after the cells were seeded into the media. THP-1+LPS+PMA cell media showed no difference from media without cells. HLF-16Lu, BEAS-2B, THP-1 and THP-1+LPS treated media had a significantly lower pH than media alone or
THP-1+LPS+PMA treated media. Over all the pH of cell treated media followed the trend: BEAS-2B < THP-1 < HLF-16Lu and THP-1+LPS < THP-1+LPS+PMA and media alone.

Figure 6.3. a) Zn\(^{2+}\) release with respect to time in SFDM with an ENM concentration of 200 \(\mu\)g/mL normalized to nanoparticle number; SEM of 5 samples. b) pH of media with and without cells present. Cells cultured for 24 hours in SFDM prior to pH reading; SEM of 3 samples. Significance is represented as # as compared to media and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

6.3.3 Cell type dependent dissolution of ZnO

Knowing that Z-MWCNT and AZO-MWCNT had variable dissolution rates in SFDM we examined how cell type effected the release of Zn\(^{2+}\) into the cell media, Figure 6.4. For all cells tested no zinc ions were detected for control, U-MWCNT, or A-MWCNT treated cells, Figure 6.4. Cells were seeded in SFDM, dosed with 14 \(\mu\)g/mL of ENM normalized to the number of U-MWCNT, and media was collected 24 hours later for testing. SFDM alone showed fewer ions present from AZO-MWCNT treatment as compared to Z-MWCNT but it was not statistically significant.
Figure 6.4: Zn$^{2+}$ release with varying cell types as compared with ion release from media alone 24 hours after ENM exposure. a) HLF-16Lu, b) BEAS-2B, c) THP-1, d) THP-1+LPS, e) THP-1+LPS+PMA were seeded in serum free media and exposed to 14 μg/mL of ENM normalized to U-MWCNT number; SEM of 5 samples. f) Graph of all cell types together to show comparison between cell lines; significance shown in Table 6.1. Significance is represented as # as compared to control, U-MWCNT and A-MWCNT and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

HLF-16Lu, BEAS-2B and THP-1 cells all caused a significant increase in the concentration of Zn$^{2+}$ as compared to Z-MWCNT and AZO-MWCNT treated media without cells present. THP-1, THP-1+LPS and THP-1+LPS+PMA cells all showed significantly less ion release from AZO-MWCNT than Z-MWCNT treatment. Ion release into the media was less for AZO-
MWCNT treated THP-1+LPS cells than ENM in media alone; this is also true for THP-1+LPS+PMA cells treated with both Z-MWCNT and AZO-MWCNT. Figure 6.4f shows a comparison between different cell types and treatments; due to limited space significance values for this figure are found in Table 6.1. The trend in concentration of $\text{Zn}^{2+}$ was similar between Z-MWCNT and AZO-MWCNT treatment with BEAS-2B > HLF-16Lu and THP-1 > THP-1+LPS > THP-1+LPS+PMA with media alone equal to THP-1+LPS and less than THP-1+LPS for Z-MWCNT and AZO-MWCNT, respectively.

Table 6.1. Significance data for $\text{Zn}^{2+}$ release when comparing all cell types. * denotes $P<0.05$, ** denotes $P<0.01$, *** denotes $P<0.001$, and NS denotes not significant.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Type</th>
<th>Z-MWCNT</th>
<th>A-MWCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLF-16Lu</td>
<td>BEAS-2B</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>HLF-16Lu</td>
<td>THP-1+LPS</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>HLF-16Lu</td>
<td>THP-1+LPS+PMA</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>THP-1</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>THP-1+LPS</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>THP-1+LPS+PMA</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>THP-1</td>
<td>THP-1+LPS+PMA</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>THP-1+LPS</td>
<td>THP-1+LPS+PMA</td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>

6.3.4 Cellular uptake of MWCNT in the THP-1 cell line

Differences were observed in the number of cells that visibly engulfed ENM, Figure 6.5. Only the THP-1 cell line was examined for this experiment. Cells were seeded in SFDM, dosed with 14 $\mu$g/mL of ENM normalized to the number of U-MWCNT and incubated for 24 hours. For this experiment MWCNT(s) were also included to determine if uptake was a function of tube length. It should be noted that THP-1+LPS+PMA were adherent to the bottom of the well while THP-1 and THP-1+LPS cells were in suspension.
When THP-1 and THP-1+LPS cells were dosed with ENM it was found that the A-MWCNT were the most avidly engulfed. AZO-MWCNT were more often found within cells than Z-MWCNT but this difference was not significant.

Figure 6.5. Percentage of total cells visibly containing MWCNT or coated MWCNT 24 hours after ENM exposure. a) THP-1, b) THP-1+LPS and c) THP-1+LPS+PMA cells were seeded in serum free media and exposed to 14 µg/mL of ENM normalized to U-MWCNT number; SEM of 100-200 cells. d) Graph of all cell types together to show comparison between cell lines. Significance is represented as # as compared to control, U-MWCNT and MWCNT(s) and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

When THP-1+LPS+PMA cells were dosed with ENM the same trend was observed but more pronounced. In this case all coated tubes had a statistically significant increase over U-MWCNT and MWCNT(s). AZO-MWCNT uptake was significantly increased over Z-MWCNT for these cells. Overall the percent of ENM uptake was similar between THP-1 and THP-1+LPS treated cells with percent uptake of U-MWCNT and MWCNT(s) < Z-MWCNT < AZO-MWCNT < A-MWCNT. This trend was maintained but the percentage significantly increased for THP-1+LPS+PMA treated cells, Figure 6.5d.
Figure 6.6: Cytotoxicity as measured by LDH release 24 hours after ENM exposure. a) HLF-16Lu, b) BEAS-2B, c) THP-1, d) THP-1+LPS, e) THP-1+LPS+PMA cells were seeded in serum free media and exposed to 14 µg/mL of ENM normalized to U-MWCNT number; SEM of 5 samples. f) Graph of all cell types together to show comparison between cell lines; negative values indicate less LDH was present in the media following treatment than in untreated cells. Significance is represented as # as compared to control, U-MWCNT and A-MWCNT and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

6.3.5 Cell type dependent cytotoxicity mirrors Zn²⁺ release

LDH release was measured for all cell-ENM combinations and graphed as percent cytotoxicity, Figure 6.6. Cells were seeded in SFDM, dosed with 14 µg/mL of ENM normalized to the number of U-MWCNT and incubated for 24 hours.
HLF-16Lu cells exposed to Z-MWCNT had a significant increase in LDH as compared to control cells. U-MWCNT and AZO-MWCNT treatment caused an insignificant rise in cytotoxicity. BEAS-2B cells exposed to both Z-MWCNT and AZO-MWCNT showed a significant increase in cytotoxicity. U-MWCNT showed a small, but not significant increase in cytotoxicity compared to control.

Both THP-1 and THP-1+LPS showed a significant increase in cytotoxicity when exposed to Z-MWCNT as compared to control cells as well as U-MWCNT and A-MWCNT treated cells. Both cell types also showed a significant decrease in cytotoxicity following exposure to AZO-MWCNT as compared to Z-MWCNT.

For THP-1+LPS+PMA LDH release from A-MWCNT, Z-MWCNT and AZO-MWCNT exposure was slightly negative when compared to control cells. From this we are unable to tell if less LDH is being released from cells with compromised membranes or if ENM exposure caused a decrease in LDH production.

Overall the trend in cytotoxicity between Z-MWCNT and AZO-MWCNT treatments was similar but commonly less severe following AZO-treatment with percent cytotoxicity in BEAS-2B > THP-1 > HLF-16Lu and THP-1+LPS > THP-1+LPS+PMA, Figure 6.6f.

To support the LDH cytotoxicity data trypan blue was also used to determine cell viability, Figure 6.7. THP-1 and THP-1+LPS trends were very similar between LDH and trypan blue assays with Z-MWCNT exposure causing more cell death than all other ENM tested. THP-1+LPS+PMA cells show very little cytotoxicity with any of the ENM treatments. It is of note that overall the cell viability for untreated control cells was lower for the THP-1+LPS+PMA treated cells with an average cell viability around 92% as compared to an average around 99% for THP-1 and THP-1+LPS cells.
Figure 6.7: Percent cell viability as measured by trypan blue uptake 24 hours after ENM exposure. a) THP-1, b) THP-1+LPS and c) THP-1+LPS+PMA cells were seeded in serum free media and exposed to 14 \( \mu \)g/mL of ENM normalized to U-MWCNT number; SEM 50-100 cells counted in 2-8 samples. Significance is represented as # as compared to control, U-MWCNT, MWCNT(s) and A-MWCNT and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.

6.3.6 **IL-1\( \beta \) protein release was dependent on cell type and MWCNT coating**

IL-1\( \beta \) protein expression was examined for all cell-ENM combinations, Figure 6.8. Cells were seeded in SFDM, dosed with 14 \( \mu \)g/mL of ENM normalized to the number of U-MWCNT and incubated for 24 hours.
HLF-16Lu cells did not produce a detectable level of IL-1\(\beta\). BEAS-2B cells did not produce a detectable level of IL-1\(\beta\) for control cells or those treated with U-MWCNT or A-MWCNT. IL-1\(\beta\) production was induced upon BEAS-2B exposure to Z-MWCNT and AZO-MWCNT to a similar degree.

**Figure 6.8:** IL-1\(\beta\) protein release into the media 24 hours after ENM exposure as measured via ELISA. a) HLF-16Lu, b) BEAS-2B, c) THP-1, d) THP-1+LPS, e) THP-1+LPS+PMA cells were seeded in serum free media and exposed to 14 \(\mu\)g/mL of ENM normalized to U-MWCNT number; SEM of 5 samples. f) Graph of all cell types together to show comparison between cell lines; comparisons within each treatment group were all *** significant unless marked with n.s. Significance in a-e is represented as # as compared to control, U-MWCNT and A-MWCNT and * for comparisons with brackets, # denotes P<0.05, ## denotes P<0.01, and ### denotes P<0.001.
THP-1 cells produced very little IL-1β protein but did display differences in ENM treatment. Both Z-MWCNT and AZO-MWCNT treatment caused a decrease in IL-1β production. THP-1+LPS cells treated with Z-MWCNT showed a significant increase in IL-1β protein over all ENM except A-MWCNT. THP-1+LPS+PMA cells produced significantly more IL-1β upon A-MWCNT exposure and significantly less upon Z-MWCNT and AZO-MWCNT exposure. IL-1β protein expression in THP-1+LPS+PMA cells was significantly suppressed for Z-MWCNT as compared to AZO-MWCNT.

In the case of IL-1β there was less of a clear trend within each ENM treatment between cell types. Overall IL-1β production was highest for THP-1+LPS and THP-1+LPS+PMA with BEAS-2B > THP-1 > HLF-16Lu.

6.4 Discussion

Through this research we sought to examine the ion release, cytotoxicity, phagocytosis, and inflammatory cytokine expression from three different cell types (fibroblasts, epithelial cells, monocytes, the latter of which were also differentiated into activated monocytes and monocyte-derived macrophages) exposed to MWCNT that were either pristine or coated with three different metal oxides (alumina, ZnO, AZO). The three different cell lines were selected to examine the pulmonary toxicity of MWCNTs coated with AZO relative to ZnO since these cell types are representative of the epithelial lining (BEAS-2B), underlying mesenchymal cells (HLF-16Lu) and monocytes/macrophages that initially uptake inhaled MWCNTs (THP-1). In particular, macrophages are the first responders to MWCNTs in the lung and trigger pro-inflammatory and pro-fibrogenic responses of epithelial cells and fibroblasts through the production of soluble mediators such as IL-1β. We have found a correlation between the concentration of Zn²⁺ in the supernatant and cytotoxicity as well as a relationship between the Zn²⁺ concentration ratio Z-MWCNT:AZO-MWCNT and IL-1β protein expression, see Table 6.2.
Previously we found that A-MWCNT suppressed fibrosis when compared to U-MWCNT in mice that were exposed via oropharyngeal aspiration and slightly elevated IL-1\(\beta\) levels in THP-1+PMA cells in vitro.\(^93\) In a similar experiment we noted that Z-MWCNT caused an acute inflammatory response after one day and similar level of fibrosis 28 days after exposure compared to U-MWCNT as well as decreased IL-1\(\beta\) protein levels in THP-1 cells in vitro.\(^{158}\)

Table 6.2. Summary of results. Zn\(^{2+}\) concentrations, cytotoxicity and IL-1\(\beta\) expression are ranked from high (1) to low (5) for each cell type. Also noted is the ratio between ZnO and AZO coated MWCNT. # denotes that the trend is not significant for either Z-MWCNT or AZO-MWCNT exposure.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>[Zn(^{2+})]</th>
<th>ZnO:AZO</th>
<th>Cytotoxicity</th>
<th>ZnO:AZO</th>
<th>IL-1(\beta)</th>
<th>ZnO:AZO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLF-16Lu</td>
<td>Fibroblast</td>
<td>2 =</td>
<td>2</td>
<td>3</td>
<td>None</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>Epithelial</td>
<td>1 =</td>
<td>1(^#)</td>
<td>3</td>
<td>3</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>THP-1</td>
<td>Monocyte</td>
<td>2 &gt;</td>
<td>2</td>
<td>3</td>
<td>Suppressed</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>THP-1+LPS</td>
<td>Monocyte</td>
<td>4 &gt;</td>
<td>2</td>
<td>2</td>
<td>Mixed</td>
<td>&gt;</td>
<td></td>
</tr>
<tr>
<td>THP-1+LPS+PMA</td>
<td>Macrophage</td>
<td>5(^#) &gt;</td>
<td>5</td>
<td>1</td>
<td>Suppressed</td>
<td>&lt;</td>
<td></td>
</tr>
</tbody>
</table>

Researchers have claimed that ions (Zn\(^{2+}\)) are responsible principally,\(^{84}\) in part,\(^{73,75,82,83}\) or not at all\(^{183}\) for the toxicity of ZnO NP. We partially attributed the differences we noted between A-MWCNT and Z-MWCNT in vivo to coating dissolution.\(^{158}\) TEM revealed the coating was still intact for A-MWCNT found within cells but the coating could not be visualized on Z-MWCNT found within cells. The question several groups have sought to answer is: where does ZnO dissolve? Three potential candidates include: dissolution occurs in the liquid lining of the lung in vivo or media in vitro before being engulfed with\(^{81}\) or without\(^{84}\) the help of secreted
factors from cells, dissolution occurs largely within cells after they are engulfed\textsuperscript{85,86} or a combination of the two.

Research has found that dissolution is time and concentration dependent; ZnO is not highly soluble without low pH or chelators.\textsuperscript{75,81} We found that at 200 $\mu$g/mL AZO-MWCNT released fewer ions with slower kinetics than Z-MWCNT which is consistent with the results of others, Figure 6.3a.\textsuperscript{72,90} To determine if ion release was a function of pH alone we also measured the pH of media from wells containing each of the cell types, Figure 6.3b. Significant differences were noted between cell lines with the pH of BEAS-2B < THP-1 < HLF-16Lu, THP-1+LPS < THP-1+LPS+PMA, the latter of which was equal to media without cells. The highest and lowest pH measurements correlate well with the concentration of Zn$^{2+}$ in solution with BEAS-2B > THP-1, HLF-16Lu > THP-1+LPS > THP-1+LPS+PMA with media alone falling in with the latter two, Figure 6.4.

In addition to the pH of the media, phagocytosis of the ENM can also lead to differences in dissolution. Uptake of CNT into monocytes and macrophages has been previously noted.\textsuperscript{93,162,196,197} ZnO NP were shown to be engulfed by RAW 264.7 macrophage cells in lysosomes. Foldbjerd et al. inspected SWCNT uptake by A549 airway epithelial cells as well as THP-1+PMA macrophage cells and found very few SWCNT within the epithelial cells compared to macrophage cells.\textsuperscript{198} BEAS-2B epithelial cells will engulf ZnO NP within caveolae readily,\textsuperscript{85,86} differences may come from the size differences between CNT and NP. Few studies have been done to examine the uptake of CNT by fibroblasts. In one such study Jin et al. showed that NIH-3T3 mouse fibroblasts will engulf DNA wrapped SWCNT.\textsuperscript{199} Fibroblasts have been known to engulf material including fluorescent beads coated with IgG or collagen but they are “nonprofessional” phagocytes unlike macrophages and monocytes and therefore not as efficient at phagocytosis.\textsuperscript{200}

Phagocytosis in this study was measured visually using a light microscope. Cells containing uncoated or coated MWCNT were calculated as a percent of total cells for the THP-1, THP-1+LPS and THP-1+LPS+PMA cells. THP-1 and THP-1+LPS cells were found to have a
similar uptake trend and percentage values with A-MWCNT uptake > AZO-MWCNT > Z-MWCNT > U-MWCNT and MWCNT(s). THP-1+LPS+PMA cells followed this trend as well but had significantly more cellular uptake than the other two cells. THP-1+LPS+PMA cells had both the highest phagocytosis of ENM along with the lowest levels of Zn$^{2+}$ in solution and highest pH. Of note, macrophages are more resistant to apoptosis than monocytes as seen in Figure 6.7 and supported by Chanput et al.$^{201}$ This means that the ENM engulfed by the more robust macrophage cells are more likely to remain within the cell as opposed to other cell types more frequently going though apoptosis and releasing their engulfed ENM and associated ions back into solution. THP-1 and THP-1+LPS cells have similar levels of phagocytosis but different concentrations of ions in solution with more ions associated with THP-1 cells. The difference in Zn$^{2+}$ was dependent on more than phagocytosis alone. One contributing factor could be that THP-1 cells have a lower pH than THP-1+LPS cells.

An interesting observation noted between the three THP-1 derived cell types was the consistent uptake of AZO-MWCNT more often than Z-MWCNT. The increase in AZO-MWCNT uptake was consistent with a decrease in Zn$^{2+}$ in solution compared to Z-MWCNT. This is also consistent with the lower ion concentration in THP-1+LPS+PMA media as these cells engulfed the most ENM of the three. Together this suggests that the more tubes that were engulfed the lower the zinc ion concentration was in the media.

The concentration of Zn$^{2+}$ in solution in media without cells following AZO-MWCNT treatment was found to be higher than in THP-1+LPS and THP-1+LPS+PMA media. It is possible that the AZO-MWCNT were engulfed before dissolution fully occurred in the cell media leading to lower Zn$^{2+}$ concentrations than ENM in media alone. The kinetics of uptake were not studied but it is possible that the differences in Zn$^{2+}$ in the supernatant between THP-1 and THP-1+LPS could be dependent on the rate of uptake and will be the focus of future study.

ENM aggregate size and crystallinity could also play a role in cellular uptake and dissolution. Z-MWCNT have the highest surface area due to their polycrystalline nature followed by AZO-MWCNT with decreased crystallinity and finally A-MWCNT which are coated amorphously,
Figure 6.1. The ENM aggregate size in SFDM follows the opposite trend with aggregates of A-MWCNT > AZO-MWCNT > Z-MWCNT. Higher surface area caused my smaller aggregates and a rougher surface could increase the rate of ion release. This trend was observed at high concentrations of ENM, Figure 6.3a, to a greater extent than at lower concentrations, Figure 6.4.

ENM cultured with THP-1, THP-1+LPS and THP-1+LPS+PMA cells enhanced the difference in ion release seen in media alone between Z-MWCNT and AZO-MWCNT and decreased the concentration of ions overall for AZO-MWCNT in the case of THP-1+LPS and both Z-MWCNT and AZO-MWCNT in the case of THP-1+LPS+PMA. Culture with HLF-16Lu and BEAS-2B cells greatly increases the ion release overall but led to no difference in ion release between coating types. In the BEAS-2B cells the concentration of Zn$^{2+}$ was high as well as cytotoxicity. This suggests one of two things: the Z- and AZO-MWCNT were engulfed at a similar rate, ions were released within the cells due to a pH change, triggered apoptosis and the release of ions or these cells secreted something into the media that enhanced the dissolution of both AZO and ZnO coatings, possibly related to the lower pH of the BEAS-2B media.

A similar response could have occurred with the HLF-16Lu cells. In this case the cytotoxicity and ion concentration was lower than that of BEAS-2B cells. If fibroblasts and epithelial cells engulfed a similar number of tubes but fewer fibroblasts went through apoptosis, as in Figure 6.6, with nanomaterial inside then fewer ions would be released. Alternatively fibroblasts could have engulfed more than epithelial cells leading to fewer Z-MWCNT and AZO-MWCNT in solution to be dissolved by factors released from the cells. A clear visualization of ENM within HLF-16Lu and BEAS-2B cells was not able to be obtained and will be the study of future work.

Differences in ion release between Z-MWCNT and AZO-MWCNT led to differences in IL-1β expression. The primary cell source of IL-1β is macrophage and endothelial cells and is associated with local and systemic inflammatory effects.\(^{78,170}\) Previously THP-1 cells without
LPS priming produced very little IL-1β but those primed with LPS produced more IL-1β overall with a significant increase following A-MWCNT treatment, PMA exposure was present in both cases. Fibroblasts respond to inflammatory cytokines like IL-1β and can also secrete IL-1β.

In this study no IL-1β was detected from HLF-16Lu fibroblasts following any treatment. BEAS-2B epithelial cells had no detectable IL-1β protein in control cells or following exposure to U-MWCNT or A-MWCNT. IL-1β protein expression was induced for BEAS-2B cells treated with Z-MWCNT or AZO-MWCNT to the same degree. This is consistent with a similar level of Zn²⁺ concentration and cytotoxicity between Z-MWCNT and AZO-MWCNT treated BEAS-2B cells.

THP-1 monocyte cells had very low, but detectable, levels of IL-1β protein with suppression of expression following the exposure to Z-MWCNT or AZO-MWCNT. No change was observed after exposure to U-MWCNT or A-MWCNT as compared to control cells. The concentration of ions in solution for THP-1 cells treated with Z- or AZO-MWCNT was different and this corresponded to a small difference in IL-1β expression in cells treated with Z-MWCNT causing more pronounced protein suppression.

THP-1+LPS activated monocyte cells secreted slightly more IL-1β protein upon exposure to A-MWCNT and slightly less upon exposure to AZO-MWCNT. U-MWCNT exposure did not change protein levels as compared to control cells. Z-MWCNT treated cells expressed significantly more IL-1β than control, U-MWCNT and AZO-MWCNT treated cells. This corresponds to differences seen in ion concentration with more ions in solution following Z-MWCNT exposure.

THP-1+LPS+PMA macrophages secreted significantly more IL-1β than the rest of the cell types. Z-MWCNT and AZO-MWCNT treated cell secreted significantly lower IL-1β than control, U-MWCNT and A-MWCNT treated cells with Z-MWCNT proteins levels
significantly lower than AZO-MWCNT treated cells. This corresponds to differences seen in ion concentration with more ions in solution following Z-MWCNT exposure.

The trend for ion concentration in solution was the same for THP-1, THP-1+LPS and THP-1+LPS+PMA cells with Z-MWCNT exposure leading to higher concentrations of Zn$^{2+}$ ions than AZO-MWCNT treatment but the change to IL-1$\beta$ expression was different. In the case of THP-1 and THP-1+LPS+PMA the differences in IL-1$\beta$ expression were similar with a more significant suppression and difference between Z-MWCNT and AZO-MWCNT treatments in THP-1+LPS+PMA cells than THP-1 cells. THP-1 cells had the highest levels of ions in solution of the three and THP-1+LPS+PMA had the lowest so it is likely not an ion concentration dependent effect. THP-1+LPS showed a different trend with Z-MWCNT exposure leasing to an increase in IL-1$\beta$ expression compared to all but A-MWCNT expression.

6.5  Summary

Previously we found that alumina coated MWCNT (A-MWCNT) suppressed fibrosis in mice when exposed via oropharyngeal aspiration when compared to uncoated (U-MWCNT). In a similar experiment we found that ZnO coated MWCNT (Z-MWCNT) caused an acute inflammatory response in mice but did not change the level of fibrosis when compared to U-MWCNT. Here we sought to determine the contribution of a small amount of aluminum to ZnO coatings (AZO-MWCNT) to toxicity. AZO-MWCNT released fewer Zn$^{2+}$ into the media when compared to Z-MWCNT without cells present. This difference was increased in media with THP-1 cells but not BEAS-2B or HLF-16Lu cells. Differences seen between ion concentrations were mirrored in cytotoxicity with higher ion concentrations correlated to increased cytotoxicity. AZO coatings were found to cause less than or equal to the amount of toxicity of ZnO coatings. Differences in ion concentrations between Z- and AZO-MWCNT were also correlated to differences in the expression of the pro-inflammatory cytokine IL-1$\beta$. All three THP-1 derived cells engulfed AZO-MWCNT more avidly than Z-MWCNT with the highest percent of uptake in THP-1+LPS+PMA cells. These same cells had the lowest
concentration of ions in solution. Neither ion dissolution within the media nor particle uptake alone is responsible for Zn\textsuperscript{2+} release and toxicity but rather a combination of both along with released factors from cells \textit{in vitro}. AZO-MWCNT would likely be less toxic than Z-MWCNT but more toxic than A-MWCNT \textit{in vivo} due to decreased ion release \textit{in vitro} as well as uptake and cytotoxicity trends shifted from Z-MWCNT towards trends for A-MWCNT.

6.6 Methods

\textit{Chemicals and Materials.} Diethylzinc (DEZ) and trimethyl aluminum (TMA) (Strem Chemicals, min 98% pure) was used as received. DEZ and TMA were co-reacted with deionized (DI) water. The reactor was purged with high purity nitrogen gas (Machine & Welding Supply Co) that was further purified with an Entegris GateKeeper located directly upstream from the reactor input. Multi-walled carbon nanotubes (MWCNT) (Helix Materials Solutions, 0.5-40 um in length) were coated as received.

\textit{MWCNT Atomic Layer Deposition (ALD).} MWCNTs were coated utilizing a method previously described.\textsuperscript{164} Briefly, approximately 40-20 mg of MWCNTs were placed into a mesh cylinder surrounded by a nonwoven polypropylene (PP) sheet (melt-blown, NC State University, College of Textiles) and secured using white, 100% cotton thread. Samples were placed into a custom made, viscous-flow, hot-walled, vacuum reactor.\textsuperscript{99,187} The reactor was kept at roughly 800 mTorr, and operated at 35 °C for ZnO deposition and 90 °C for alumina and alumina doped ZnO (AZO). For ZnO and alumina deposition either DEZ or TMA was introduced into the reactor and held by closing all ports into and out of the reactor for 60 seconds; this allowed for proper diffusion of the precursor through the PP. The reactor was then purged with N\textsubscript{2} gas. This was followed by a co-reacting step with DI water that was also allowed to be held in the reactor for 60 seconds. Alumina coatings were applied with 82 TMA/water cycles. ZnO coatings were applied with 50 DEZ/water cycles. To create AZO coated MWCNT (AZO-MWCNT) first one ALD cycle of alumina was coated onto the tubes. Following that 13 cycles of ZnO ALD were applied. This was repeated four times with one
final layer of ZnO on the outside for a total of 4 alumina layers and 53 ZnO layers, see Figure 6.1e.

Short MWCNT were created through extended sonication in ethanol using the parameters and sonicator described in the next section. The key difference was that the sample was sonicated for 10 seconds and then allowed to cool for 30 seconds. This was repeated for 48 hours. Following sonication the sample was left open in a hood to allow the ethanol to evaporate.

**MWCNT Characterization.** The thickness of the ALD layers coating the MWCNT were measured using a JEOL 2000Fx scanning transmission electron microscope (TEM). TEM samples were prepared by dropping 3 µl of ENM (engineered nanomaterials) suspended in 100% ethanol on to a carbon faced TEM grid (Protochips) and allowed to dry in air. Vials of ENM were suspended using a cuphorn sonicator (Qsonica) at room temperature immediately preceding dosing using 7 amps, 50 W for a total energy of 2910 J on average. From TEM images the length of the ENM were also measured. ImageJ software was used to measure MWCNT length and ZnO thickness.

Mass gain of the MWCNT after the ALD coating process was measured (Fischer Scientific, accuSeries-accu124) to both validate that the nanomaterial was being coated and also for the potential to correct for this weight change when dosing to normalize to the number of MWCNT dosed instead of the total weight.

Dynamic light scattering (DLS, Malvern Zetasizer ZSP) was used to determine the MWCNT aggregate size and zeta potential. ENM were suspended in 0.1% pluronic F-68 (Sigma-Aldrich) and sonicated as described above and then diluted to a concentration of 40 µg/mL in serum free media (SFDM, F-12K Nutrient Mixture with albumin solution and ITS; Gibco, Sigma- 35% in DPBS, and Lonza respectively). Samples were allowed to settle for 1 hour before measurements were taken. Diameter values were reported using the number percent of the diameter measured. Three different samples were used to establish significance.
Zn$^{2+}$ concentrations were measured with a NanoMolar Zinc Assay Kit (ProFoldin) according to manufacturer’s instructions with the addition of a 5 minute centrifuge step to all samples at 14,000 rpm before the start of the assay. ENM were incubated in serum free media in the dark at 37 °C at a concentration of 200 μg/mL to establish the release of ions with respect to time. Fluorescence was read using a FLUOstar Omega (BMG Labtech). Zn$^{2+}$ was also measured from the supernatant of cells incubated with ENM for 24 hours. Values represent measurements taken from five different samples.

The crystal structure of the ZnO coated MWCNT (Z-MWCNT) as compared to the AZO-MWCNT was determined using x-ray diffraction (XRD, Rigaku SmartLab). Peaks for both the MWCNT and ZnO were measured.

**Cell Culture and Dosing.** Human monocytes (THP-1) (ATCC) were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Gibco) and kept at 37 °C with 5% CO$_2$. Human lung fibroblasts (HLF-16Lu, ATCC) as well as lung epithelial cells (BEAS-2B, ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and kept at the same incubator conditions. THP-1 cells were examined with three different culturing conditions: SFDM with no additions, with the addition of 10ng/mL lipopolysaccharide (LPS, Sigma Aldrich) or with the addition of LPS and 5 nM phorbol 12-myristate 12-acetate (PMA, Sigma Aldrich). LPS was administered just prior to plating and dosing cells. PMA was introduced to THP-1 flasks 1 hour prior to seeding cells. Cells were transferred into 24 well plates (Becton Dickinson) for experimentation at a concentration of approximately 4.5x10$^5$ cells/mL for THP-1 cells and 80-90% confluency for HLF-16Lu, BEAS-2B cells and THP-1+LPS+PMA. Cells were plated in serum free media (SFDM) (F-12K Nutrient Mixture with albumin solution and ITS) (Gibco, Sigma- 35% in DPBS, and Lonza respectively). The concentration range of MWCNT used to dose cells in vitro was consistent with that previously established through intra-laboratory consortium testing of engineered nanomaterials.$^{203}$ Cells were dosed with a stock solution (10 mg/mL) of engineered nanomaterials (ENM, in this case MWCNT that were either uncoated or coated in ZnO, alumina or AZO) in sterile 0.1% pluronic F-68 (Sigma-Aldrich). The dose of U-MWCNTs, A-
MWCNT, Z-MWCNT and AZO-MWCNT were 14, 31.6, 40 and 37.5 µg/mL, respectively. The ALD coated tubes were dosed at a concentration normalized to achieve a dose with as similar a number of nanoparticles as possible to the uncoated tubes. To adjust this the mass gain following ALD was utilized in equation 1:

\[
MW\text{CNT dose} \times \frac{m_{\text{coated \ MWCNT}}}{m_{\text{uncoated \ MWCNT}}} = \text{coated \ MWCNT dose}
\]

Eqn. 1

Where “m” is the mass of the nanomaterial in grams and the dose is in µg/mL. In this case the value for the mass of coated MWCNT divided by the mass of uncoated MWCNT for this experiment was 2.3, 2.8, and 2.7 for A-MWCNT, Z-MWCNT and AZO-MWCNT, respectively. From this the 14 µg/mL U-MWCNT dose was converted to a 31.6, 40 µg/mL, and 37.5 for A-MWCNT, Z-MWCNT and AZO-MWCNT dose, respectively.

**Cell Viability.** Cell viability was determined using a 0.4% Trypan Blue solution (Life Technologies) according to the manufacturer’s protocol. Briefly, Trypan Blue was mixed 1:1 with the THP-1 or THP-1+LPS cell suspensions and the number of living and dead cells was counted using a hemocytometer. Between 50 and 150 cells were counted in three or more wells. Cells viability was also measured using a lactose dehydrogenase assay (LDH, Thermo Fisher) according to the manufacturer’s instructions with the addition of a 5 minute centrifuge step to all samples at 1,000 rpm before the start of the assay. Supernatant was centrifuged for 5 minutes at 1000 rpm for 5 minutes prior to running the LDH assay to remove ENM from solution. Values represent measurements taken from five different samples.

**Percent of MWCNT uptake.** THP-1, THP-1+LPS and THP-1+LPS+PMA cells were exposed to ENM. Twenty four hours later 10 µLTHP-1 and THP-1+LPS cells were removed from suspension and placed onto a glass slide. Cells were counted that visibly contained ENM at 40X under an Olympus BX40 light microscope. THP-1+LPS+PMA cells were adherent and thus required a different protocol for visualization. Small round glass cover slips were cleaned in 100% ethanol and washed 3 times with phosphate buffered saline. Slips were placed in the bottom of the 24 well plate and cell containing media was added. Slips were removed 24 hours
after treatment and the percent of cells containing ENM were counted. For each condition 100-200 cells were counted in 2-3 different wells.

*ELISA.* IL-1β protein levels in the cell media were measured via ELISA (DuoSet, R&D Systems). Samples were assayed according to manufacturer instructions with the addition of a 5 minute centrifuge step to all samples at 14,000 rpm before the start of the assay. Absorbance was read at 450 nm by a microplate spectrophotometer (Multiskan EX, ThermoFisher Scientific) with a correction wavelength of 540 nm. Values represent measurements taken from five different samples.

*pH.* pH was measured using a SevenEasy pH meter (Mettler Toledo). Cells were incubated for 24 hours and then the media was removed. Values represent measurements taken from three different samples.

*Data and Statistical Analysis.* Data and statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software Inc.). A one-way ANOVA with a post hoc Tukey test was used to determine significance between samples. When noted an unpaired two tailed t-test was used to determine significance. A significance of p<0.05 was used unless otherwise noted. Data values were expressed as mean ± SEM.

6.7 **Acknowledgements**

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Figure A.1. Zn$^{2+}$ ion concentration in serum-free defined medium (SFDM) incubated with uncoated MWCNT (U-MWCNT) or ZnO-coated MWCNT (Z-MWCNT) in the absence of cells or in SFDM from THP-1 cell incubated with U-MWCNTs or Z-MWCNTs (SFDM + THP-1). 40 µg/mL of Z-MWCNTs or 14 µg/mL U-MWCNT were dosed into SFDM with or without THP-1 cells for 24 hours. Data are the mean +/- SEM of 6 replicate determinations.
Figure A.2. Dose-dependent decrease in cell viability of THP-1 cells 24 hours after exposure to U-MWCNTs, Z-MWCNTs, or ZnO nanoparticles (NP). Cell viability was measured by Trypan Blue staining. Data represents the average of living cells from a total of 100 to 300 cells per dose and treatment.
Figure A.3. Lung sections stained with Masson’s trichrome from mice 28 days after exposure to saline pluronic (control), U-MWCNTs or Z-MWCNTs. Collagen is indicated by blue stain (arrows). TB (terminal bronchiole), ADB (alveolar duct bifurcation, AD (alveolar duct).
Figure A.4. Lung collagen levels in mice following oropharyngeal aspiration of Z-MWCNT or U-MWCNTs. Right lung lobes were collected at 1 and 28 days and collagen measured by Sircol assay. Collagen levels were normalized to total lung protein in each sample. Each treatment group (control, U-MWCNT, Z-MWCNT) contained 3, 4, and 4 animals at one day, respectively and 4, 5, and 5 animals at 28 days, respectively. No significant differences were observed between treatment groups at either time point.
Figure A.5. TGF-β1 and OPN mRNA levels in lung tissue from mice exposed to Z-MWCNTs or U-MWCNTs. Each treatment group (control, U-MWCNT, Z-MWCNT) contained 3, 4, and 4 animals at one day, respectively, and 4, 5, and 5 animals at 28 days, respectively. *P<0.05 compared to control.
Figure A.6. Survival of mice following oropharyngeal aspiration of Z-MWCNT (Z-MW) or U-MWCNT (MW). At one day post-exposure mice treated with Z-MW were lethargic and exhibited shivering, while mice exposed to MW were asymptomatic. Each treatment group (control, U-MWCNT, Z-MWCNT) contained 4, 5, and 5 animals, respectively.