Characterizing Effects of Nitric Oxide Sterilization on tert-Butyl Acrylate Shape Memory Polymers

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Abstract

As research into the potential uses of shape memory polymers (SMPs) as implantable medical devices continues to grow and expand, so does the need for an accurate and reliable sterilization mechanism. The ability of an SMP to precisely undergo a programmed shape change will define its ability to accomplish a therapeutic task. To ensure proper execution of the in vivo shape change, the sterilization process must not negatively affect the shape memory behavior of the material. To address this need, this thesis investigates the effectiveness of a benchtop nitric oxide (NOx) sterilization process and the extent to which the process affects the shape memory behavior of a well-studied tert-Butyl Acrylate (tBA) SMP.

Quantifying the effects on shape memory behavior was performed using a two-tiered analysis. A two-tiered study design was used to determine if the sterilization process induced any premature shape recovery and to identify any effects that NOx has on the overall shape memory behavior of the foams.

Determining the effectiveness of the NOx system—specially, whether the treated samples are more sterile/less contaminated than untreated—was also performed with a two-tiered analysis. In this case, the two-tiered analysis was employed to have a secondary check for contamination. To elaborate, all of the samples that were deemed not contaminated from the initial test were put through a second sterility test to check for contamination a second time.

The results of these tests indicated the NOx system is an effective sterilization mechanism and the current protocol does not negatively impact the shape memory behavior of the tBA SMP. The samples held their compressed shape throughout the entirety of the sterilization process. Additionally, there were no observable impacts on the shape memory
behavior induced by NOx. Lastly, the treated samples demonstrated lower contamination than the untreated.

This thesis demonstrates the effectiveness of NOx as a laboratory scale sterilization mechanism for heat triggered shape memory polymers. The shape memory analysis indicated that the magnitude of the length changes induced by NOx is small enough that it does not make a statistically significant impact on the shape memory behavior of the foams. Additionally, there were no observable effects on the shape memory behavior induced by NOx. The results further indicated the NOx system is effective at sterilizing porous scaffolds, as none of the sterilized samples showed contamination. Testing methods proved to be effective because the initial sterility test was able to identify all of the contaminated samples and preliminary results indicated that NOx sterilization improves the sterility of the foams.
Characterizing Effects of Nitric Oxide Sterilization on \textit{tert}-Butyl Acrylate Shape Memory Polymers

by

Ben Phillippi

B.S., Chemical Engineering, Ohio University, 2014

Thesis
Submitted in partial fulfillment of the requirements for the degree of Master’s of Science in Bioengineering

Syracuse University
May 2017
I want to dedicate this to the loving memory of my grandfathers, Walt Phillippi and Duane Siekkinen...I miss you
Acknowledgements

First and foremost I’d like to thank my advisor, Dr. James Henderson for his patience, support and guidance these past three years. Dr. Henderson has been instrumental in my growth as an independent researcher by instilling in me the value of prospective statistics based experimental design. Dr. Henderson was also pivotal in my personal growth, helping me to set project goals and achieve them by sharing his vast knowledge.

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Schemes</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Synopsis</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Shape Memory Polymers</td>
<td>1</td>
</tr>
<tr>
<td>1.2.1 Background and Current Applications in Regenerative Medicine</td>
<td>1</td>
</tr>
<tr>
<td>1.2.2 Defining Shape Memory Behavior</td>
<td>3</td>
</tr>
<tr>
<td>1.2.3 Challenges Associated with Sterilization of SMPs</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Sterilizing with Nitric Oxide (NOx)</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Scope of Thesis</td>
<td>5</td>
</tr>
<tr>
<td>1.5 References</td>
<td>6</td>
</tr>
<tr>
<td>Chapter 2: SMP Fabrication and NOx Sterilization Protocol</td>
<td>11</td>
</tr>
<tr>
<td>2.1 Synopsis</td>
<td>11</td>
</tr>
<tr>
<td>2.1 Background and Motivation</td>
<td>11</td>
</tr>
<tr>
<td>2.2 SMP Preparation</td>
<td>13</td>
</tr>
<tr>
<td>2.2.1 SMP Compression</td>
<td>14</td>
</tr>
<tr>
<td>2.3 NOx Sterilization Protocol Improvements</td>
<td>16</td>
</tr>
<tr>
<td>2.3.1 Background</td>
<td>16</td>
</tr>
<tr>
<td>2.3.2 Protocol Improvements</td>
<td>17</td>
</tr>
<tr>
<td>2.4 Conclusion</td>
<td>19</td>
</tr>
<tr>
<td>2.5 References</td>
<td>21</td>
</tr>
<tr>
<td>Chapter 3: Quantifying the effects of NOx Sterilization Process on Shape Memory Behavior</td>
<td>27</td>
</tr>
<tr>
<td>3.1 Synopsis</td>
<td>27</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>27</td>
</tr>
<tr>
<td>3.3 Experimental Design</td>
<td>29</td>
</tr>
</tbody>
</table>
### Table of Contents

#### Chapter 3: Results Analysis

- 3.3.1 Manual Shape Memory Analysis ............................................. 29
- 3.3.2 Automated Shape Memory Analysis ....................................... 29
- 3.3.3 Statistics ............................................................................. 30

#### Chapter 4: Effectiveness of NOx Sterilization

- 4.1 Synopsis ................................................................................. 44
- 4.2 Introduction ........................................................................... 44
- 4.3 Material and Methods ............................................................ 45
  - 4.3.1 Experimental Design ....................................................... 45
  - 4.3.2 Growth Media Preparation ............................................ 46
  - 4.3.3 Agar Plate Preparation .................................................... 46
  - 4.3.4 Statistics ........................................................................ 46
- 4.4 Results ................................................................................... 46
- 4.5 Discussion ............................................................................ 47
- 4.6 Conclusion ............................................................................ 48
- 4.7 Acknowledgments ................................................................ 48
- 4.8 References ............................................................................ 48

#### Chapter 5: Conclusions and Future Directions

- 5.1 Conclusions .......................................................................... 52
- 5.2 Recommendations for Future Work ....................................... 54
  - 5.2.1 NOx System and Protocol Developments ....................... 54
  - 5.2.2 Shape Memory Analysis ............................................... 55
  - 5.2.3 Sterility Analysis ............................................................. 56
- 5.3 References ............................................................................ 57

VITA........................................................................................................ 58
List of Tables

Table 3-1. Comparing average strain loss values across every group which with the exception of the 50-control group, contained six sets of length measurements. The standard deviations are inconsistent and often much larger than the actual strain loss value. However, due to the magnitude of the strain loss averages, these results do not indicate a problem for real world applications of this system.................................................................34

Table 3-2. Comparing average unrecovered strain values across every group in the study. Once again, large standard deviations indicate a lack of precision in detecting the magnitude of length changes. However, it is because the magnitude is so small that these results indicate a negligible loss in strain which is a positive for effectiveness of the sterilization process.............................................35

Table 3-3. Comparing average values for fixing ratio across every group in the study. Each group had six samples and some of the samples were less than 5mm in diameter. There were no observable differences with each group having an average of at least 1.0. Standard deviations indicate a high degree of precision given the measurements were conducted under dynamic mechanical analysis.36

Table 3-4. Comparing average values for recovery ratio across each group in the study. Once again the differences between groups are negligible with every recovery ratio starting at 99%. .................................................................37
List of Schemes

Scheme 3-1. Four length measurements taken at different points during the sterilization process. These variables were used to adapt the equations for fixing and recovery ratios into ones better suited for describing the NOx system. For example take a 50% sterilized sample: after cutting the $L_1$ measurement is taken and it would be 5.0 mm. Next the foam is compressed using the hot press method and the $L_2$ measurement is taken and would be expected at 2.5 mm. The foam is then sterilized and after the 24 hours of aeriation has completed the $L_3$ measurement is taken and would be expected to read 2.55 mm. The final step is to trigger shape recovery in the foam by heating to 70°C and then allowing it to cool down to room temperature before taking the $L_4$ measurement. The foam would be expected to fully recover the magnitude of its initial shape so $L_4$ would be expected to read 5 mm.

Scheme 4-1. Graphical representation of the obvious contamination test results. Abbreviations represent compression ratio and whether or not the sample was sterilized (i.e. 50-S refers to 50% compression ratio and sterilized). Shaded boxes identify samples that showed gross contamination after spending 48 hours in a heated shaker.
List of Figures

Figure 1-1. One-way shape memory cycle for a typical tBA foam SMP (solid black line).

Starting at (i) and traveling to the right: the sample is deformed at a constant temperature above its $T_{\text{Trans}}$. Next the temporary shape is fixed into the sample by lowering the temperature and holding the stress constant. While holding the temperature constant, the stress on the sample is then unloaded. The final step is to trigger shape recovery by heating the sample above its glass transition temperature.

Re-used with permission from [16]. .........................................................10

Figure 2-1. X-Ray radiograph showing what appears to be a bacterial cist (denoted with an asterisk) forming around the Sleeve/Allograft group. Interestingly, the Sleeve/Allograft group was the only one to show this type of contamination when the graft and sleeves were sterilized using the same NOx based technique. Compared to the grafts, the sleeves do have smaller pore sizes which could explain the differences or it could be that airborne bacteria adheres to the sleeve more readily than the graft during surgery. Re-used with permission from [1]. .........................................................22

Figure 2-2. Preliminary evidence that NOx affects the glass transition of temperature of porous SMPs. The sharper void shown in the top line will affect how well the material transitions through shape memory. Re-used with permission from [2]. .................................................................23
Figure 2-3. Preliminary foam compression method. Involved manually compressing and measuring each foam simultaneously which turned out to be time consuming and inaccurate. Samples would have a tendency to become dislodged during compression because the applied force was non-uniform. Additionally, samples would prematurely recover their uncompressed shape because there was no step for precisely fixing the temporary compressed shape.

Figure 2-4. Layout for final compression system. Uniform compression and complete temporary shape fixing could be applied to four foams in much faster time as compared to the older protocol. Once the system was heated to between 65-70°C, force was be applied until the foams were barely touching the top portion of the press. The entire system was then held in place for five minutes to fully relax the polymer chains. Lastly after the five minutes had elapsed, force was applied to completely close the press and the heating mechanism was turned off for 15 minutes. This allowed the internal temperature of the foams to go well below their T_{\text{Trans}} which precisely locks in the temporary shape.

Figure 2-5. A picture that showcases the components and layout of the NOx sterilization system. Troubleshooting experiments indicated the NO inlet tube must be connected during the vacuum step to avoid any leaks. Additionally the vacuuming step, house air, and humid air additions all are done through the “valve for humid air” shown above. Re-used with permission from [2].
**Figure 3-1.** Graphically comparing strain loss values. Standard deviations are inconsistent and often times larger than the magnitude of the strain loss. Due to the magnitude of strain loss means across all groups, this indicates a positive outcome for the NOx system.

**Figure 3-2.** Graphically comparing strain loss values with adjusted axis to showcase the large standard deviations. Once again these do not represent negative outcomes due to the magnitude of the strain loss values.

**Figure 3-3.** Graphically showcasing the negligible differences in unrecovered strain. The size of the standard deviations indicate the amount of unrecovered strain is below the level of detection for the assay. And the magnitudes of unrecovered strain loss indicate a positive outcome for the NOx.

**Figure 3-4.** Graphically comparing average values for fixing ratio. There were no observable differences due to every group having an average value of 1.0. Each reported value is the average of three one-way shape memory cycles.

**Figure 3-5.** Graphically comparing average values for recovery ratios. These results were just as positive with each value starting at 99%. Each reported value is the average of three one-way shape memory cycles.

**Figure 4-1.** Results of batch 3 obviousness test. Contaminated samples were noticeably darker than the rest of the samples and their media had increased turbidity. Pictured above with a black arrow is the only contaminated sample in this batch so it would not move onto the second tier streak test.
Figure 4-2. Differences between a contaminated agar plate and a sterile one. The initial batch 1 test (shown on the left) was excluded from the final analysis and a second batch 1 test (pictured on the right) was conducted. The results from the initial batch 1 test were excluded because sterilized samples showed obvious contamination and every agar plate from the streak test indicated gross contamination.
Chapter 1: Introduction

1.1 Synopsis

This chapter presents an overview of shape memory polymers along with their potential uses in biomedical applications. Shape memory behavior is defined, and potential challenges associated with sterilizing shape memory polymers are presented. The motivation for nitric oxide (NOx) as a sterilization mechanism is presented. Lastly, the chapter concludes with an outline of the studies completed to achieve the goals of the thesis.

1.2 Shape Memory Polymers

1.2.1 Background and Current Applications in Regenerative Medicine

Shape memory polymers (SMPs) are a class of materials that have the ability to undergo a mechanical shape change when initiated by a “trigger” mechanism. More specifically, the shape change occurs after a series of events take place: first a permanent shape is fixed into the material by crosslinking the polymer chains (this step is often completed during fabrication), next a temporary shape is fixed into the material by immobilizing the polymer chains through a process such as cooling, and lastly the shape change is triggered by reversing the immobilization step. Established trigger mechanisms include electricity, solvent interactions, thermal heating, and exposure to light, pH, or magnetic fields [1-8]. For example, the SMP used in this study is a heat triggered amorphous polymer, which means the shape changing events take place below or above the glass transition temperature (T_{\text{Trans}}).

Research into shape memory polymers and their potential uses in biomedical applications is rapidly expanding (readers interested in applying SMPs in vitro or in vivo are invited to examine...
sources [16-27] for a thorough background into these areas), but, for the scope of this thesis, only recent discoveries with uses as medical devices or tissue engineered constructs will be highlighted (specifically highlighting implantable devices is purposefully done to remain in-line with the overarching goal of establishing an *in vivo* focused sterilization process that can aid in accomplishing the therapeutic task). SMPs represent an attractive option for implantable medical devices due in large part to the flexibility and precision of the shape changing events. Researchers have a high degree of control over how the material behaves *in vivo*. Additionally, with the use of the temporary shape fixing, there is the potential for surgeries to be less invasive and complex [10].

*Tert*-butyl acrylate (tBA) SMPs, the model SMP employed in the present study, have been widely studied with positive results. To further illustrate that point, the following innovations are designed around tBA or use a foam architecture. Maitland and colleagues developed a laser activated SMP foam that is triggered to fill gaps in a blood vessel that occur during an aneurysm [12]. Similarly, Metcalf et al. have developed a cold hibernated elastic memory foam that was successfully able to dissipate the aneurysm in a dog model [13]. For other cardiovascular applications, Yakaki et al. was able to show that a tBA based SMP can be used as a stent [14].

The ability of these devices to accomplish their therapeutic task depends on how well the sterilization process meets the challenges associated with sterilizing complex three dimension shapes. These challenges include not inducing any premature shape recovery (effectively not interfering with the shape changing trigger mechanism), not damaging the chemical structure of the material, and being able to sterilize the material in such a way that allows it to retain its implantable shape while not inducing any adverse immune responses *in vivo*. As such, this thesis investigates the effects of a novel nitric oxide (NO) based sterilization technique on the shape memory behavior and sterility of a tBA based SMP.
1.2.2 Defining Shape Memory Behavior

Determining how NOx affects the shape memory behavior of the tBA SMP system is a significant part of this thesis, so first the methods behind the analysis must be defined. Analyzing and quantifying shape memory behavior (data analysis in Chapter 3) is commonly accomplished with a dynamic mechanical analyzer [9]. One-way shape memory is a well-known technique for analyzing shape memory behavior and as previously stated the following steps are involved (Figure 1-1): at a temperature above $T_{\text{Trans}}$ the sample is uniaxially deformed to a desired strain, the stress is then held constant and the temperature is lowered below $T_{\text{Trans}}$ in order to fix the temporary shape. While staying below $T_{\text{Trans}}$ the stress is unloaded, and, finally, shape recovery is triggered by increasing the temperature above $T_{\text{Trans}}$.

Quantifying shape memory behavior is completed through the use of two variables, fixing and recovery ratios.

The equations for each of these variables are as follows:

\[
R_F(N) = \frac{\varepsilon_u(N)}{\varepsilon_M(N)} \times 100\% \tag{Eq 1-1}
\]

\[
R_r(N) = \frac{\varepsilon_u(N) - \varepsilon_p(N)}{\varepsilon_u(N) - \varepsilon_p(N-1)} \times 100\% \tag{Eq 1-2}
\]

In these equations $N$, $\varepsilon_u$, $\varepsilon_M$, and $\varepsilon_p$ represent the cycle number, strain after unloading, strain before unloading, and the final strain after heating and unloading.

1.2.3 Challenges Associated with Sterilization of SMPs

Any potential uses for SMPs as implantable tissue constructs revolve around the precise shape change of the material. Depending on the material and the surgical process, the SMP could be sterilized in its permanent or temporary shape. However, there are significant challenges
involved with sterilizing SMPs. A potential challenge is the shape of the material because SMPs can be bulky or awkward shapes making it difficult to sterilize the entirety of the material. Additional challenges include heat and solvent sensitivity. For these reasons conventional sterilization processes are not ideal for SMPs because the shape memory behavior of the material would be hindered or damaged.

Conventional laboratory sterilization mechanisms include autoclaving, UV light, and ethylene oxide. Autoclaving is a sterilization process that involves exposing the samples to high-pressure steam. Autoclaving takes place at a temperature that is much higher than the $T_{Trans}$ of most materials and therefore is not useful for sterilizing SMPs. UV sterilization involves exposing samples to UV light in an enclosed space for a given amount of time. UV is not always an effective method for three-dimensional materials especially when there is a large z-axis and the process could affect the material properties. Ethylene Oxide (EtO) is an effective room temperature sterilization process where samples are exposed to EtO with controlled temperatures and humidity. The process has drawbacks in the required additional safety protocols and longer processing cycles due to the pre-sterilization treatments. EtO is highly reactive and harmful to humans, which, combined with the necessity of adding higher concentrations to sterilize various materials, makes the process difficult to implement on a laboratory scale.

1.3 Sterilizing with Nitric Oxide (NOx)

Nitric Oxide has the potential to fulfill all of the requirements needed to sterilize SMPs. The strong oxidizing potential of NO is what allows it to be an effective sterilization mechanism because it can cleave amide bonds, which degrades bacterial DNA. The sterilization process works by reacting Nitric Oxide (NO) with air to form Nitrogen Dioxide (NO$_2$), which reacts again with water to form Nitric Acid (HNO$_3$) [15]. Nitric Acid has anti-microbial properties, which will hopefully keep the SMP sterile for further uses. To recap, major benefits of NOx sterilization are
that it takes place at room temperature, can adequately handle complex three dimensional shapes, safe to use in large quantities, does not require pre-sterilization treatment steps, and it adds additional antimicrobial properties to the material. While NOx does offer significant improvements for sterilizing thermally sensitive SMPs, there are some aspects of the protocol that need further investigation.

The sterilization mechanism for the NOx process is the strong oxidizing potential of NO gas, and this could be a potential problem if the sterilant has any adverse effects on the chemical structure of the material. Additional underdeveloped steps in NOx sterilization protocols are the process controls. Specifically the amounts of NO and O\textsubscript{2} to add during the process have not been well established. Based on the overall sterility reaction, it is clear that air is added in excess compared to NO but the exact amounts may be different for each material. Other important process controls to measure and study are the system humidity and temperature of humid air because both of them have the potential to trigger premature shape recovery. In an effort to reduce variability in the sterilization process, the present study aims to establish a refined protocol that can be used to study how NOx affects the shape memory behavior and sterility of the tBA SMP system.

1.4 Scope of Thesis

This thesis examines the effects of NOx based sterilization on the shape memory behavior and sterility of a tBA based SMP. The aim of this work is to quantify any adverse effects induced by NOx while developing protocol improvements that will aid in future uses of the NOx system. A chapter-by-chapter outline is presented below.

The current chapter (Chapter 1) provides an overview of shape memory polymers, the challenges associated with sterilizing them, and the characteristics that make NOx a good candidate to sterilize heat sensitive SMPs. Chapter 2 discusses the NOx system updates developed to improve the functionality of the NOx system. Additionally, background information on previous
uses and experiments with the NOx system are presented to serve as motivation for investigating the shape memory behavior and sterility.

**Chapter 3** begins the data analysis sections and, specifically, details a two-part shape memory analysis. Manual shape memory analysis was done to determine if the SMPs could hold their compressed shape throughout the sterilization cycle. Automated analysis was done to compare shape memory data before and after sterilization to determine any adverse effects of NOx sterilization. **Chapter 4** presents the results of a preliminary two-tiered sterility analysis. The two-tiered analysis consisted of an initial bacterial growth test where sterilized samples were placed in a test tube with bacterial growth medium and contamination judgements were based on obvious changes to the media color and turbidity. The second tiered of the sterility analysis was a streak test where samples that were not obviously contaminated had an aliquot of medium distributed on an agar plate and bacteria was given a second chance to grow. Finally, **Chapter 5** discusses the overall conclusions of the thesis along with suggestions for future work.

### 1.5 References


Program download at: http://henderson.syr.edu/downloads/


Figure 1-1. One-way shape memory cycle for a typical tBA foam SMP (solid black line).

Starting at (i) and traveling to the right: the sample is deformed at a constant temperature above its $T_{\text{Trans}}$. Next the temporary shape is fixed into the sample by lowering the temperature and holding the stress constant. While holding the temperature constant, the stress on the sample is then unloaded. The final step is to trigger shape recovery by heating the sample above its glass transition temperature. Re-used with permission from [16].
Chapter 2: SMP Fabrication and NOx Sterilization Protocol

2.1 Synopsis

This chapter presents the methods developed in this thesis to prepare and sterilize SMPs. Prior, foundational work on NOx sterilization as well as relevant prior findings related to the effect of NOx on SMPs are presented and discussed. Design goals, such as improved pressure control, reduction in the opportunity for leaks, increased control of humid air handling, and development of improved and more uniform mechanical testing, are identified and the means for addressing these goals presented. Finally, the NOx system and associated protocol developed in light of this prior work is presented.

2.1 Background and Motivation

Research into the potential uses of SMPs as implantable tissue constructs to combat various diseases and injuries is rapidly expanding. One specific application for SMPs is to replace bone grafts in the treatment of segmental bone defects. A recent publication was able to show how a two-tiered SMP system can be deployed to fill and stabilize a segmental defect in a mouse model [1]. The materials in the two-tiered system consisted of an expanding tBA based porous scaffold (material studied in this thesis) to fill the defect and a polyurethane based SMP sleeve to contract around the tBA scaffold ensuring defect stability.

Each portion of the SMP system was sterilized with NOx because the researchers needed to sterilize the tBA scaffold in its temporary compressed form and shape recovery would be triggered if the process was not conducted at room temperature. The state of the NOx system and protocol will be discussed in greater detail in Section 2.3 but as it applies to its uses in the segmental defect study, there was only a rough working protocol. To explain, the researchers did not know the exact amount of reactants to add during the process and they were not aware of any
pressure leaks occurring during the sterilization cycle. These problems were due to the pressure gauge on the system being too large to read the system pressure. The underdeveloped portions of the sterilization protocol may have impacted the results of the segmental defect study.

The test groups in the segmental defects study were separated by what was used to fill the defect site and they are as follows: allograft, sleeve/allograft, and SMP graft [1]. The results of the segmental defect study indicated there were problems with the sterility of the sleeves because x-rays of the implant site in the sleeve only group revealed what appeared to be bacterial cists forming around the implant (Figure 2-1) [1]. Given that NOx was the sterilization mechanism for the sleeves in this study, questions were raised in regards to the efficacy of the protocol with porous materials. In a separate publication completed by one of the authors after the segmental defect study, preliminary results indicated that NOx sterilization affects the glass transition temperature of the sleeves (Figure 2-2) [2, Appendix 2].

Evaluating all of the background knowledge regarding the NOx sterilization system and experiments using it provides the motivation for the current study design. In regards to the porous tBA scaffold, there is a need to be able to sterilize it in its compressed form and there were no preliminary studies to determine how NOx affects its shape memory behavior. In regards to the sleeves, there was evidence that NOx adversely affects its shape memory behavior and there was evidence that the NOx system could have problems sterilizing highly porous samples. Taking these results into consideration, this work aimed to fill in gaps on how NOx affects the shape memory behavior of the tBA scaffold (hence-forth referred to as a foam) by conducting experiments with various amounts of pre-sterilization compression ratios. Additionally, while the results of the segmental defect study indicate that the NOx system may have more problems with sterilizing the polyurethane sleeves versus the tBA foams, it was still important to gather additional evidence that shows the efficacy of the NOx system.
2.2 SMP Preparation

As previously described, fabricating the tBA foams involves combining acrylate chemistry with a modified porogen leaching technique [1, 3-4]. Briefly, the first step is to combine tBA and butyl acrylate (BA) monomers at a 92-8 (tBA-BA) wt% ratio to ensure the glass transition temperature of the material is at or close to body temperature. The next steps were to add the remaining reactants, which were tetraethyleneglycol dimethacrylate crosslinker at 5 wt% of the monomer weight and 2,2-dimethoxy-2-phenylacetophene photoinitiator at 1 wt% of the monomer weight. The entire solution was then added to a fused salt template.

Constructing a fused salt template is a crucial step in the overall SMP fabrication process because it ensures the proper amount of interconnectivity and mechanical strength. Making templates consists of coating a 20 mL glass vial in Rain-X so the salt will form a puck and avoid sticking to the vial. Next, the vials are placed in a capped Styrofoam cooler with a beaker full of warm water for 24 hours. The final step is to dry the templates for 24 hours in a vacuum oven. Once dry, the tBA/BA solution is evenly distributed among each template and the reaction is initiated by curing the foams in UV light for two hours.

After curing, the foams go through a series of washing steps to remove unreacted salt and monomers. The first washing step is carried out by placing the foams in a deionized water bath inside of a heated shaker to allow for unreacted salt to precipitate out. Water was changed approximately every 24 hours with foams spending a total of 48 hours in the shaker. The second washing step is designed to remove unreacted monomers and is done by placing the foams in a beaker filled with methanol. The foams spent 24 hours soaking in methanol and then another 12 hours drying under a fume hood before moving to a vacuum oven to remove any excess methanol. After the washing steps had concluded, the next step was to cut out smaller foam samples from the larger templates.

Downstream processing steps were designed around having foams with 5mm diameters so this preparation step was paramount to the overall protocol. Preparing the experimentally sized
foams started with placing the larger template in a 70 °C isothermal oven for 30 seconds to relax the polymer chains and make it possible to cut out smaller samples. Using a 5mm biopsy punch a series of smaller foams were punched out from the template. Heating and then applying a significant amount of force to the templates resulted in 5mm foams that have a highly compressed shape. The internal strain brought on by having this compressed shape must be relieved before proceeding, to prevent premature shape recovery during sterilization. After each 5mm foam has been cut and allowed to cool to room temperature, the initial strain is relieved by placing them back into a 70 °C isothermal oven for approximately 30 seconds. Finally after one more cooling step, the foams were cut to 5mm in length and then moved to the compression portion of the protocol.

2.2.1 SMP Compression

As mentioned in the introduction, primary goals for this thesis were to study how NOx affects the shape memory behavior of the foams, sterility of the foams, and how effective is the NOx system at sterilizing foams with varying degrees of compressed pore sizes. Meeting all of these goals meant designing experiments for the shape memory and sterility assays that included test groups with varying amounts of compression ratios. Accurately applying different amounts of compression to different samples meant a new compression system would have to be developed.

In the initial stages of these studies, it became apparent that the current compression method affected the accuracy of each investigative assay and in its current state was not useful. Showcased in Figure 2-3, the system involved taping two pieces of Teflon to a pair of calipers to make as flat a surface as possible. Then based on a 5mm starting length of the foams, the new compressed length would be calculated (i.e. 50% compression meant the final length would be 2.5mm) and the foam would be placed into the calipers. Now the entire system would be inserted into an isothermal oven at 70°C for approximately 1 minute to relax the polymer chains and prepare
the sample for compression. The next step would be to apply as much force as possible to achieve the desired length of the sample. Often times this was not easy for larger compression ratios and samples had a tendency to become dislodged from the calipers. Finally once the desired length had been met, the sample would be withdrawn from the oven and allowed to cool down to room temperature in order to fix the new temporary shape. This process was time consuming in that only one sample could be compressed at a time, inaccurate because the compression was far from uniform across the surface of the sample, and in total this adversely affected both the sterility and shape memory assays.

One method for analyzing shape memory involved detecting changes in the length of the sample that were on the scale of hundreds of a millimeter and this would not be possible without complete uniform compression. Results of the sterility assay were also affected because the samples would not be able to hold their compressed shape through the entirety of the sterilization process.

Improving the compression system would then improve the accuracy of each investigative assay. The new compression system was designed around efficiency and the ability to provide uniform compression. Pictured in Figure 2-4 the system was the combination of a hot press and aluminum spacers. Spacers were sheets of aluminum cut to specific thicknesses with four 5 mm holes to fit four foam samples for every test group. Spacer thicknesses were 3.6 and 2.35 mm respectively which equates to 28 and 53% compression ratios given a 5mm initial length of the sample.

To briefly illustrate how the process works consider preparing a 28% compression group: first the 3.6 mm spacer is placed on the hot press. The hot press is then heated to between 65-70 °C and once it reaches this temperature, four 5 mm foams are inserted to the holes. Now manual force is applied until the foams are barely touching the top portion of press and then the entire press is held in place for five minutes. This step serves to fully relax the polymer chains and make
the final compression easier. After the five minutes had elapsed, additional force was applied until the press was completely closed. Now with the press closed the heating mechanism for the hot press is turned off and the foams are held in place for approximately 15 minutes. This allows the temperature to reach 20 ºC, which precisely fixes the temporary shape because 20ºC is below the T_{Trans} of the tBA foams. Throughout the entire data collection portions of both investigative assays, this system proved to be reliable and meet all of the necessary standards.

2.3 NOx Sterilization Protocol Improvements

2.3.1 Background

To understand the changes made to the sterilization protocol and system, a closer examination of the older protocol as it was published is in order. Figure 2-5 shows the NOx system configuration and a summary of the older protocol is as follows [2, Appendix 2]: seal the samples in a Tyvek sterilization pouch, fold the pouch in half and place it in the sample chamber so the gas soluble side is facing inwards, add a small unknown amount of NO gas, then add another small unknown amount of NO along with house air (“house” denotes from air-line in laboratory), and finally after 1 hour add 120 mL of humid air.

Humid air is paramount to the success of the process because it facilitates the reaction that increases the sterility of the samples. Humidity was generated by placing a glass beaker in an 80 ºC isothermal oven for 30 minutes. After 30 minutes, warm water is added to the beaker and the humid air begins to form. Finally after 1 hour has passed since the NO and O_2 additions, 120 mL of humid air is added to the sample chamber. 18 hours after the humid air additions the pouches are withdrawn from the chamber and allowed to aerate for 24 hours before being used. Each step in this protocol was important to maintain functionality of the NOx system but each step also needed improvements to maximize the potential of NOx as a sterilization mechanism.
The general principles of the protocol proved to be a solid backbone, but as mentioned in the introduction of this chapter, there was some evidence that the system and protocol were not functioning as well as they could be. This lead to a slight protocol modification, which was to vacuum the sample chamber for three minutes prior to the additions of NOx and O₂. The first experiments with the addition of the vacuuming step indicated negative results because the materials used in the experiment underwent premature shape recovery. These experiments did not include tBA/BA foams but they did indicate further modifications were in order to ensure the materials were able to hold their shape throughout the entirety of the process.

It was at this point that the idea of adding small amounts of NO and O₂ mentioned above was implemented because that would limit the amount of unreacted NO in the chamber. Implementing the vacuum and small NO/O₂ additions were important modifications because the rationale behind each step either directly or indirectly addressed problems identified within the NOx system. Vacuuming out the sample chamber to begin the process creates a pressure differential that aids in NOx penetrating through each sample. Adding small amounts of NO and O₂ pushed the system reaction toward the desired product of nitric acid and it also limited the availability of NO in the chamber to possibly damage the chemical structure of the materials.

2.3.2 Protocol Improvements

Previous experiments with the NOx system indicated the theory behind the system was good but significant improvements were necessary before it could be a reliable sterilization mechanism. Basic operation of the NOx system was improved when a smaller vacuum pressure gauge was installed. This allowed for the system pressure to be tracked throughout the entire process which in itself was beneficial because different amounts of NO and O₂ could be added in different experiments to determine the optimal amounts.
Installing a new pressure gauge was helpful but it also revealed some problems. The first major problem was a leak; the system was unable to hold pressure throughout the process regardless if it was under vacuum or positive pressure. Revealing the source of the leak was accomplished by soaking all of the junctions surrounding the NO and air inlets in soapy water so that when air was added to the system, there would be bubbles coming from the source of the leak. This trick revealed the source of the leak to be an uncovered threaded junction right below the air inlet valve. Wrapping the junction in Teflon tape and performing the soapy water test again indicated the leak was fixed.

Pressure was now held throughout the sterilization cycle until a new leak was discovered during the humid air additions. These additions involved using a 12 mL syringe to withdraw humid air from a beaker with heated water and transferring it to the sample chamber. Multiple solutions were attempted and they included using a new syringe, replacing the stopper on top of the air inlet valve, and trying different size needles. As it turns out, the problem was the 12 mL syringe itself.

Using a 12mL syringe was the problem because transferring the maximum volume of the syringe meant a momentary flexion in the body, which loosened the seal between the syringe and the 22 gauge needle attached to it. This was confirmed to be the problem when an empty 12 mL syringe was attached to the air inlet valve the system pressure remained constant. With that knowledge the solution to the problem was simply swapping the 12mL syringe for a 24mL. An additional improvement was made to the humid air step, and that was how humidity was generated.

In previous studies the humid air was generated by heating an empty glass beaker, adding water to it, and waiting. No temperature measurements were taken throughout this process, rather simply feeling the temperature of the beaker was used to determine when it was appropriate to add to the sample chamber. The reproducibility of this step was improved by generating humid air with a hot plate and taking temperature measurements of the air inside. Approximately 150 mL of deionized water was added to a capped 250 mL Erlenmeyer flask with a stir bar and the entire
flask was placed on a hot plate with the temperature set to 30 °C. Temperature measurements of the air inside the beaker were taken throughout the hour long process that the humidity was being generated. Unfortunately due to malfunctions in the thermometer, accurate temperature measurements were unable to be obtained for every experiment and for that reason they are left out of the final protocol. Not being able to directly measure the temperature of the humid air being added to the system is cause for slight concern, but the protocol for generating the humidity has a much higher degree of reproducibility and therefore is deemed to be an improvement. Combining all of these improvements lead to the development of a refined and detailed protocol.

At this point the NOx system was functioning well throughout the entirety of a sterilization cycle. A summary of the improved protocol is as follows: cut out 3 in long sterilization pouches for every test group (a maximum of 3 pouches were used for each sterilization cycle due to the size restrictions of the sample chamber), fold the pouches in half so when they are placed in the sample chamber the gas soluble side is facing inward. Next the sample chamber is vacuumed down to -20 mmHg (-15 PSI g) because of the limitations of the house vacuum line. The remaining steps are to add the reactants and in order they are: add 1 mmHg NO, add 6-8 mmHg house air, add 1-2 mmHg NO, and finally add house air until the pressure is equilibrated at 0 PSI. Begin heating water to 30 °C in a capped beaker and begin taking air temperature measurements if possible. After 1 hour has passed since the last NO addition, begin adding 120 mL of humid air using a 24 mL syringe. After humid air additions the system pressure could range from 0-6 PSI and there was no observable differences on the effectiveness of the protocol when the system pressure was in that range.

2.4 Conclusion

When the NOx system was initially built there were some flaws but overtime the appropriate modifications were made. Initial sterilization experiments revealed changes needed to be made to the working protocol to aid the ability of the sterilant gas to penetrate through materials
with reduced pore sizes and to push the overall sterility reaction toward the desired product. These modifications were made in the form a vacuum step to begin the process and small additions of each reactant added after the vacuum step.

General system improvements including a vacuum pressure gauge, 24 mL syringe, and fixing gas leaks with Teflon tape immediately improved the accuracy and precision of all experiments using the NOx system. More specific protocol developments such as the hot press compression, amounts of NO and house air added during the process were tuned to sterilizing the tBA foams and could possibly be changed for future uses.

With all of these improvements, the working protocol proved to be reliable but not perfect. Three different steps in the protocol either induced premature shape recovery or are believed to and will need improving. These steps are the amount of NO added during the sterilization cycle, temperature of the humid air and humidity of the house air added towards the beginning of the cycle. Humidity of the house air is not currently measured but experiments conducted during the summer months had a much larger issue with premature shape recovery. The total amount of NOx added to the system was measured to be 2-3 mmHg and when the amount added was much greater than this, the sterilization pouches became stained with a deep yellow color. Foams did not always prematurely recover their shape but it happened enough times that this step is highlighted as requiring additional focus. Mentioned previously was that the inability to obtain consistent temperature measurements of the humid air before it was added to the sample chamber and focusing improvements on this step will drastically increase the effectiveness of the entire system. While these controls are not perfect, concentrating work to reduce variability in these steps will lead to massive improvements in the overall system.
2.5 References


Figure 2-1. X-Ray radiograph showing what appears to be a bacterial cist (denoted with an asterisk) forming around the Sleeve/Allograft group. Interestingly, the Sleeve/Allograft group was the only one to show this type of contamination when the graft and sleeves were sterilized using the same NOx based technique. Compared to the grafts, the sleeves do have smaller pore sizes which could explain the differences or it could be that airborne bacteria adheres to the sleeve more readily than the graft during surgery. Re-used with permission from [1].
Figure 2-2. Preliminary evidence that NOx affects the glass transition of temperature of porous SMPs. The sharper void shown in the top line will affect how well the material transitions through shape memory. Re-used with permission from [2].
Figure 2-3. Preliminary foam compression method. Involved manually compressing and measuring each foam simultaneously which turned out to be time consuming and inaccurate. Samples would have a tendency to become dislodged during compression because the applied force was non-uniform. Additionally, samples would prematurely recover their uncompressed shape because there was no step for precisely fixing the temporary compressed shape.
Figure 2-4. Layout for final compression system. Uniform compression and complete temporary shape fixing could applied to four foams in much faster time as compared to the older protocol. Once the system was heated to between 65-70°C, force was be applied until the foams were barely touching the top portion of the press. The entire system was then held in place for five minutes to fully the relax the polymer chains. Lastly after the five minutes had elapsed, force was applied to completely close the press and the heating mechanism was turned off for 15 minutes. This allowed the internal temperature of the foams to go well below their T_{trans}, which precisely locks in the temporary shape.
Figure 2-5. A picture that showcases the components and layout of the NOx sterilization system. Troubleshooting experiments indicated the NO inlet tube must be connected during the vacuum step to avoid any leaks. Additionally the vacuuming step, house air, and humid air additions all are done through the “valve for humid air” shown above. Re-used with permission from [2].
Chapter 3: Quantifying the effects of NOx Sterilization Process on Shape Memory Behavior

3.1 Synopsis

This chapter presents the methods used to determine how NOx sterilization affects the shape memory behavior of tBA foams. Established shape memory variables (Fixing and Recovery Ratios) were used to complete an automated shape memory analysis. A second shape memory analysis was completed by adapting the equations for fixing and recovery ratios to better describe the NOx system. Lastly, it is shown that NOx does not have an adverse effect on the shape memory behavior of the foams.

3.2 Introduction

As previously mentioned, the viability of the NOx system as a reliable form of sterilization will be characterized in part by how it impacts shape memory behavior. The final characterization will be made by completing two separate shape memory analysis (manual and automatic). The goal of the manual analysis is to measure the length changes of the compressed samples before and after sterilization. Measuring length changes allows for shape memory to be quantified and how NOx effects the ability of the foams to hold their shape through the sterilization cycle which is important for surgical procedures. The goal of the automated analysis was to compare the fixing and recovery ratios before and after sterilization. While different in their primary goals, each assay relied on accurate quantifications of their data pieces.
To manually quantify the data, four length measurements were conducted at different points in the sterilization process (Scheme 1). The length measurements were then used to adapt the equations for fixing and recovery ratios which originally are [1]:

\[ R_F(N) = \frac{\varepsilon_u(N)}{\varepsilon_M(N)} \times 100\% \]  \hspace{1cm} (Eq 3-1)

\[ R_r(N) = \frac{\varepsilon_u(N) - \varepsilon_P(N)}{\varepsilon_u(N) - \varepsilon_P(N-1)} \times 100\% \]  \hspace{1cm} (Eq 3-2)

In these equations \( N, \varepsilon_u, \varepsilon_M, \) and \( \varepsilon_P \) represent the cycle number, strain after unloading, strain before unloading, and the final strain after heating and unloading. These equations were the basis for the automated analysis. For the manual analysis, fixing ratio was replaced by strain loss \( (\varepsilon_{\text{Loss}}) \) and recovery ratio was replaced by unrecovered strain \( (\varepsilon_{\text{Unrecovered}}) \). The variables are represented by the following two equations:

\[ \varepsilon_{\text{Loss}} = \frac{L_3 - L_2}{L_2} \]  \hspace{1cm} (Eq 3-3)

\[ \varepsilon_{\text{Unrecovered}} = \frac{L_4 - L_1}{L_1} \]  \hspace{1cm} (Eq 3-4)

To re-iterate the information found in Scheme 1, \( L_1 \) is the initial uncompressed length of the sample, \( L_2 \) is the pre-sterilization compressed length, \( L_3 \) is the post-sterilization compressed length, and \( L_4 \) is the post-sterilization uncompressed length.

The aim of this study was mainly to gather the necessary data to prove the effectiveness of the NOx system that was indicated in previous studies [2]. Additionally it was important to study how the amount of pre-sterilization fixed strain affected the shape changes and recovery of the material because a possible future use of this material is to be an implanted bone graft. And in that case, it would be paramount that the material recover only when triggered and be able to hold its compressed shape until that time.
3.3 Experimental Design

3.3.1 Manual Shape Memory Analysis
The layout of this study was designed around having a reliable process that could impart a specific amount of compression into each foam sample. As previously discussed in Chapter 2, the answer was building a series of hot plate “spacers” that were cut to a given thickness, which allows for a fixed uniform strain to be imparted into the samples. Spacer thicknesses were 3.6 and 2.35 mm respectively which equates to 28 and 53% given a 5mm initial length of the sample. Results will be presented and discussed that reference 25 and 50% groups; the numbers were simplified solely for explanation purposes.

To accurately test the effects of the NOx system on foams with shrunken pore sizes, the following test groups were used in both investigative assays: 0% control, 0% sterilized, 25% control, 25% sterilized, 50% control, and 50% sterilized. Each group contained two technical replicates meaning each sterilization pouch contained two samples, one with a small black sharpie dot to differentiate the replicates. Lastly, three independent replicates were observed giving a total of six sets of length measurements to complete the manual shape memory analysis (one of the 50%-control samples was misplaced so that group only had five samples).

3.3.2 Automated Shape Memory Analysis
Similarly to the manual shape memory analysis, the test groups consisted of foams with between 0-50% compression ratios. One sample from each independent replicate was used to establish baselines (denoted “Pre-Sterilization” on all figures) because that allowed for proper comparison to determine the effects of NOx sterilization on the shape memory behavior of the samples. Each sample went through three cycles of one-way shape memory analysis and a total of
three independent replicates with one technical replicate were studied for this assay (some of the samples in the assay were smaller than 5 mm in diameter).

3.3.3 Statistics

Statistical analysis was performed using two sample t-tests for means because the goal of the study was to determine effects of a “treatment”, and all of the samples from each independent replicate originated from the same batch. Comparisons between control and sterilized groups, and different compression amounts compared to each other (i.e., 25% control vs. 50% control) were deemed to be statistically significant based on the benchmark of $P < 0.05$.

3.4 Results

3.4.1 Manual Shape Memory Analysis

To characterize how sterilizing with NOx affects shape memory behavior, length measurements were conducted before and after sterilization on groups that ranged from 0-50% pre-sterilization fixed strain. Measurements were then used to adapt well known shape memory variables into equations better suited to describe the system used in the study. Beginning with strain loss, it can be seen that changes induced by NOx are below the level of detection for the assay (Table 3-1 and Figure 3-1). Averages strain loss values ranged from -2 to 2% and the standard deviations were often twice as large as the initial value (Figure 3-2). Values for unrecovered strain followed a similar trend in being very small (Figure 3-3). Across both control and sterilized groups, the averages for unrecovered strain ranged from -3.6 to 2% with large standard deviations (Table 3-2). For both variables the statistical analysis indicated no difference between control vs. sterilized, and no difference between all but one of the groups, which was the
25% control group for the strain loss variable. This group was statistically significant from the 0 and 50% control groups used to calculate the strain loss variable.

3.4.2 Automated Shape Memory Analysis

Three cycles of one-way shape memory analysis were used to quantify the fixing and recovery ratios before and after sterilization for groups of samples ranging from 0-50% pre-sterilization compression.

Beginning with fixing ratio, there were negligible differences between every group (Figure 3-4). Averages for all groups including the baseline data were 1.0 with standard deviations on the order of 1.0x10^{-4} \% (Table 3-3). Recovery ratios followed a similar trend showing no differences between the all of the groups tested (Figure 3-5). All groups tested indicated an average of at least 99\% with standard deviations on the magnitude of 1.0x10^{-3} \% (Table 3-4). Finally, the paired two sample tests used to complete the statistical analysis indicated no significant differences between control vs. sterilized and between different compression groups.

3.5 Discussion

In this study two different forms of shape memory analysis were conducted to quantify how exactly sterilizing with NOx impacts the SMPs. Beginning with the manual analysis, it was found that NOx does not induce significant changes in the shape memory behavior of the samples. While the size of the standard deviations reported in this section indicate significant variability in the results, the magnitude of the actual strain values would not be problematic when applied to downstream applications. For example, the average strain loss and unrecovered strain for the 50\% sterilized group was 2.3 \pm 4.5\% and 0.91 \pm 0.88\% respectively (Table 3-1, 3-2). Taking the high end of these spectrums would not change the practicality or effectiveness of these samples to be used as part of a treatment for segmental bone defects.
Moving onto the automated analysis, the results indicated there are no observable impacts from sterilizing with NOx. Both fixing and recovery ratios were found to be 99+% across all groups tested (Tables 3-3, 3-4). Additionally, the precision of the dynamic mechanical analysis provided small standard deviations ranging from $1 \times 10^{-3}$ to $1 \times 10^{-4}$%. Judging by the results of each shape memory analysis, the NOx system appears to function quite well and this is true when extra attention is given to a couple steps in the protocol.

An in-depth review of the NOx system and working protocol can be found in chapter 2. However as it applies to the results reported in this chapter, there are some specific steps that could induce premature recovery. Preliminary work in this study revealed the importance of three steps in the working protocol: amount of NOx added during sterilization cycle, temperature of humid air added during concluding steps of sterilization cycle, and the humidity of the house air added during the sterilization cycle. Each of these steps were found to be the cause or believed to be the cause of premature shape recovery in the foams.

### 3.6 Conclusion

The results presented in this study indicate that there is no measureable or negative impact on the shape memory behavior of the tert-butyl acrylate SMPs used in these experiments. Shape memory was analyzed manually to characterize how the sterilization process affects the SMPs ability to retain its compressed shape and recover back to its original uncompressed form. While the variability of these particular results was high, the actual values of strain loss and unrecovered strain were low enough to indicate the NOx system does not negatively impact shape memory behavior of the samples.
Automated shape memory analysis was conducted under dynamic mechanical analysis and indicated similar results to that of the manual assay. Before and after sterilization, the fixing and recovery ratios of the SMPs were observed to be at least 99% for all groups. In summary, given the results and statistical analysis presented in this chapter I can say that the current working protocol for NOx sterilization will not negatively impact the shape memory behavior of the specific tert-butyl acrylate SMPs used in the study.

3.7 References


**Table 3-1.** Comparing average strain loss values across every group, which with the exception of the 50-control group, contained six sets of length measurements. The standard deviations are inconsistent and often much larger than the actual strain loss value. However, due to the magnitude of the strain loss averages, these results do not indicate a problem for real world applications of this system.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Compression Ratio (%)</th>
<th>Mean ± St. Dev (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>-2.76 ± 2.96</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>-0.46 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.31 ± 1.84</td>
</tr>
<tr>
<td>Sterilized</td>
<td>0</td>
<td>0.37 ± 2.23</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.77 ± 1.05</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.33 ± 4.53</td>
</tr>
</tbody>
</table>
Table 3-2. Comparing average unrecovered strain values across every group in the study. Once again, large standard deviations indicate a lack of precision in detecting the magnitude of length changes. However, it is because the magnitude is so small that these results indicate a negligible loss in strain which is a positive for effectiveness of the sterilization process.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Compression Ratio (%)</th>
<th>Mean ± St. Dev (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>-2.76 ± 2.96</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.91 ± 2.18</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.35 ± 0.41</td>
</tr>
<tr>
<td>Sterilized</td>
<td>0</td>
<td>0.37 ± 2.23</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>-3.62 ± 5.08</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.91 ± 0.89</td>
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</table>
Table 3-3. Comparing average values for fixing ratio across every group in the study. Each group had six samples and some of the samples were less than 5mm in diameter. There were no observable differences with each group having an average of at least 1.0. Standard deviations indicate a high degree of precision given the measurements were conducted under dynamic mechanical analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Compression Ratio (%)</th>
<th>Mean ± St. Dev (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Sterilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.000193 ± 2.73x10^-4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.000365 ± 5.17x10^-4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.000280 ± 3.96x10^-4</td>
</tr>
<tr>
<td>Sterilized</td>
<td>0</td>
<td>1.000433 ± 6.13x10^-4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.000504 ± 4.28x10^-4</td>
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<tr>
<td></td>
<td>50</td>
<td>1.000382 ± 5.41x10^-4</td>
</tr>
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<td></td>
<td></td>
<td>1.000243 ± 3.44x10^-4</td>
</tr>
</tbody>
</table>
Table 3-4. Comparing average values for recovery ratio across each group in the study. Once again the differences between groups are negligible with every recovery ratio starting at 99%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Compression Ratio (%)</th>
<th>Mean ± St. Dev (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Sterilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1.00746 ± 1.22x10⁻³</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.9985 ± 1.42x10⁻³</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.9982 ± 1.39x10⁻³</td>
</tr>
<tr>
<td>Sterilized</td>
<td>0</td>
<td>0.9963 ± 3.45x10⁻³</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.9997 ± 4.96x10⁻³</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.9972 ± 4.92x10⁻³</td>
</tr>
</tbody>
</table>
Scheme 3-1. Four length measurements taken at different points during the sterilization process.

These variables were used to adapt the equations for fixing and recovery ratios into ones better suited for describing the NOx system. For example take a 50% sterilized sample: after cutting the $L_1$ measurement is taken and it would be 5.0 mm. Next the foam is compressed using the hot press method and the $L_2$ measurement is taken and would be expected at 2.5 mm. The foam is then sterilized and after the 24 hours of aeration has completed the $L_3$ measurement is taken and would be expected to read 2.55 mm. The final step is to trigger shape recovery in the foam by heating to 70°C and then allowing it to cool down to room temperature before taking the $L_4$ measurement. The foam would be expected to fully recover the magnitude of its initial shape so $L_4$ would be expected to read 5 mm.
Figure 3-1. Graphically comparing strain loss values. Standard deviations are inconsistent and often times larger than the magnitude of the strain loss. Due to the magnitude of strain loss means across all groups, this indicates a positive outcome for the NOx system.
Figure 3-2. Graphically comparing strain loss values with adjusted axis to showcase the large standard deviations. Once again these do not represent negative outcomes due to the magnitude of the strain loss values.
**Figure 3-3.** Graphically showcasing the negligible differences in unrecovered strain. The size of the standard deviations indicate the amount of unrecovered strain is below the level of detection for the assay. And the magnitudes of unrecovered strain loss indicate a positive outcome for the NOx.
**Figure 3-4.** Graphically comparing average values for fixing ratio. There were no observable differences due to every group having an average value of 1.0. Each reported value is the average of three one-way shape memory cycles.
Figure 3-5. Graphically comparing average values for recovery ratios. These results were just as positive with each value starting at 99%. Each reported value is the average of three one-way shape memory cycles.
Chapter 4: Effectiveness of NOx Sterilization

4.1 Synopsis

This chapter presents the results of a preliminary sterility assay that indicates sterilizing with NOx is an effective treatment. Sterility tests were completed in two-tiers by first testing for obvious contamination by adding samples to bacterial growth medium and allowing possible bacteria to grow. The second tier of the tests was executed with samples that did not show obvious contamination and that entailed taking a small portion of the growth media and allowing it grow on an agar plate for at least 48 hours. Both tiers of the test indicated positive outcomes in that no treated samples showed obvious contamination.

4.2 Introduction

At this stage in the development process of the NOx system, it was important to have a qualitative check on the system. Additionally, it was important to know how if any the amount of pre-sterilization fixed strain affected the sterility of the samples. Answering both of these questions came by designing a two-tiered sterilization assay that used different amounts of fixed strain as the test groups.

A two-tiered sterility assay was employed to provide an extra degree of confidence in the results. To elaborate, the initial “obviousness” test involved placing sterilized and unsterilized samples in a test tube with bacterial growth media and allowing contamination to grow. Contamination was subjectively judged based on drastic changes in the color and clarity of the growth media. The second tier of the sterility assay was a streak test and it involved taking the non-contaminated samples from the initial test and giving any contamination a second chance to proliferate. Being able to double-check the results of the first sterility test validated the experimental method and results of the overall sterility assay.
The goal of this study was to not only test the effectiveness of sterilizing with NOx but also to develop the methods for future tests. Being able to accurately test the sterility of a different material type will be beneficial for future projects.

4.3 Material and Methods

4.3.1 Experimental Design

To be in line with the major goals of the study, the experimental design revolved around test groups with various amounts of compression ratios. For this study there were a total of three independent replicates (with one technical replicate) and each replicate contained the following groups: 0% control, 0% sterilized, 25% control, 25% sterilized, 50% control, 50% sterilized, and a media only control was added for the initial obviousness test. After treatment, these samples were added to 3 mL of Luria-Bertani (LB) medium and placed in a heated shaker at 37°C and 100 RPMs. Test tubes were examined for contamination 2-3 times every 24 hours and when contamination initially appeared, the second tier of the assay was scheduled. It should be noted that between the judgment of initial contamination and execution of the second tier of the assay, there was a 24-48 hour grace period to allow for any additional samples to develop contamination. Although that never occurred, it was important to add this extra time to be absolutely sure in the initial contamination judgment. The second and final tier of the study (commonly denoted as a streak test) involved testing the samples that were not obviously contaminated. An aliquot of growth medium (approximately 100 µL for test samples and 200 µL for media control) was pipetted onto an agar plate and allowed to grow in an incubator for at least 48 hours. For clarification; none of the samples were pre-inoculated with bacteria, this study was only testing for environmental contamination.
4.3.2 Growth Media Preparation

For this study a well-known bacterial growth medium called Luria-Bertani (LB) was used to test for obvious contamination. The medium was prepared by combining 5 g of Tryptone, 2.5 g of Yeast Extract, and 500 mL of distilled water. Autoclaving was the final step to insure the media was sterile before being used in the assay.

4.3.3 Agar Plate Preparation

Streak testing was used as a secondary sterility test for this study. The agar plates for the tests were prepared by combining 5 g of Tryptone, 2.5 g of Yeast Extract, 5 g of NaCl, 7.5 g of BactoAgar, and 500 mL of distilled water. From there the flask had to be stirred, and autoclaved before 20 mL of agar medium was distributed to each plate for testing.

4.3.4 Statistics

Due to the limitations of small sample size and non-numerical results, the statistical analysis was carried out using non-parametric statistics. A non-parametric analysis regarding how to estimate the proportion of a population based on samples was the basis of the statistical analysis [1]. This turned out be effectively what was done in this study; determine the proportion of controlled and sterilized samples that are contaminated post treatment.

4.4 Results

To determine if sterilizing with NOx is an effective method, a two-tier sterility assay was conducted. Samples ranging from 0-50% pre-sterilization fixed strain went through the sterilization protocol and then were added to a test tube containing 3 mL of bacterial growth media. Each test tube was then placed in a heated shaker at 37°C and 100 RPM so any bacteria present in the sample could have the opportunity to grow. Samples were then subjectively judged to be contaminated based on whether or not the color and clarity of the growth media had drastically
changed. Non-contaminated samples were then put through a streak test to account for the possibility that during the initial contamination test the concentration of bacteria in the sample was not large enough to change the color of the media.

Beginning with the initial obviousness test, the only contaminated samples occurred in replicates 2 and 3 (denoted Batches on Scheme). Both compressed controls were contaminated in batch 2 and only the 25%-C group was contaminated in batch 3 (Scheme 4-1). This means that all seven samples from batch 1, five samples from batch 2, and six samples from batch 3 were all going to complete a streak test. Across all batches the streak tests indicated no contaminated samples which indicates the obviousness test is sufficient to detect contamination. Finally, the statistical analysis was confined to only the controlled group (along with limitations listed in section 4.3.4) because none of the sterilized samples showed contamination. And as previously mentioned, the data was analyzed as how to determine a proportion of a population based on samples. Therefore I estimate that the rate of contamination in untreated samples would be 49.5 ± 16.6%.

4.5 Discussion

In this study a two-tier sterility assay was carried out to ensure the functionality of the NOx system remained intact after all of the adjustments discussed in Chapter 2. Beginning with the obviousness test, none of the treated samples indicated contamination. To clarify how contamination was judged, a photograph taken during the assay is presented below with an arrow indicating the only contaminated sample (Figure 4-1). Moving on to the streak test, none of the samples used in the final analysis indicated contamination. An example to illustrate how contamination was judged in this portion of the assay is also presented below (Figure 4-2).

Taking all of this into consideration, this study gives a positive outcome for the sterilization system. Previous work indicated that sterilizing compressed samples might purpose an issue and
show contamination due to the inability of the NOx to penetrate through the shrunken pore sizes. However, the present study indicated that up to 50% fixed strain is not an issue. While these results are promising, more experiments need to be conducted with these materials to lower the variability and expanding the type of material or growth media will lead to a better understanding on how effective the NOx system can be.

4.6 Conclusion

The results presented in this study indicate that NOx is an effective mechanism for sterilization. Sterility was analyzed with a two-tier assay that contained an initial obviousness test and a secondary streak test. Results from each tier of the assay indicate positive outcomes in that no treated samples showed obvious contamination. Specifically there were no compressed sterilized samples that showed contamination which indicates the current protocol allows the NOx to penetrate through the sample. Additionally the two-tier method for analyzing sterility proved to be effective but not immune to error. In conclusion, this preliminary study provided favorable results that indicate the uses of NOx as an effective reproducible sterilization mechanism should continue to be explored.

4.7 Acknowledgments

This study was completed in large part due to the collaboration with Dr. Dacheng Ren and his laboratory. The Ren lab provided materials and facilities to conduct the streak tests. Lastly, I would like to thank Drs. Geetika Sanjay Choudary and Fangchao Song for devoting so much time in training and helping me to design and execute this study.

4.8 References

**Scheme 4-1.** Graphical representation of the obvious contamination test results. Abbreviations represent compression ratio and whether or not the sample was sterilized (i.e. 50-S refers to 50% compression ratio and sterilized). Shaded boxes identify samples that showed gross contamination after spending 48 hours in a heated shaker.
Figure 4-1. Results of batch 3 obviousness test. Contaminated samples were noticeably darker than the rest of the samples and their media had increased turbidity. Pictured above with a black arrow is the only contaminated sample in this batch so it would not move onto the second tier streak test.
Figure 4-2. Differences between a contaminated agar plate and a sterile one. The initial batch 1 test (shown on the left) was excluded from the final analysis and a second batch 1 test (pictured on the right) was conducted. The results from the initial batch 1 test were excluded because sterilized samples showed obvious contamination and every agar plate from the streak test indicated gross contamination.
Chapter 5: Conclusions and Future Directions

5.1 Conclusions

The goal of this thesis was to characterize and quantify the effects of NOx sterilization on the shape memory behavior and sterility of a tBA/BA porous scaffold. Building on previous work that established the potential of NOx as a sterilization mechanism and established the proper fabrication technique for the SMP studied in this thesis [1], NOx system and protocol improvements were developed to aid in accurate data collection.

In Chapter 2, background information on the effects of NOx on a polyurethane SMP sleeve along with previously published sterilization protocols were discussed to provide the motivation behind new protocol improvements. General system improvements included installing a vacuum pressure gauge capable of reading the system pressure throughout the sterilization cycle, identifying and fixing pressure leaks, increasing the size of the syringe used to transfer humid air to the system from 12 mL to 24 mL, and generating humid air in a more reproducible way by placing a capped beaker on a hot plate. Protocol improvements developed specifically for this thesis include a foam compression system that involved combining a hot press with aluminum spacers to impart an accurate and uniform compression. General NOx system improvements were developed to increase the effectiveness, basic operation of the system, and should continue to be used throughout its lifetime. Protocol developments specific to this thesis including the hot press compression system and amounts of NO and O$_2$ added during the sterilization cycle were designed to improve the sterility of the tBA/BA foams and could possibly be adapted to different materials.

In Chapter 3, a two-tier shape memory analysis was conducted to quantify how sterilizing with NOx affects the shape memory behavior of tBA/BA foams. The first analysis was done manually and involved taking four length measurements of the compressed foams before and after sterilization. These measurements were used to adapt formulas for fixing and recovery ratios which are commonly accepted variables for quantifying shape memory behavior, into strain loss and
unrecovered strain which are better suited to describe the performance of the NOx system. Using the newly developed hot press compression system, foam samples with 0, 25, and 50% compression ratios had their lengths measured before and after sterilization. Beginning with strain loss during sterilization, averages ranged from -2 to 2% with standard deviations ranging from 0.11-4.5%. These values indicate that any shape recovery induced by NOx is below the level of detection for the assay. Additionally even if these spectrums are observed at the high end, the magnitude of the strain loss is low enough that it would not be a significant problem in regards to downstream applications.

The automated shape memory analysis was completed using a dynamic mechanical analyzer and quantified using fixing and recovery ratios. Groups with 0, 25, and 50% compression ratios were put through three cycles of one-way shape memory analysis after sterilization to compare to baseline data acquired by putting an unsterilized and uncompressed sample through three cycles of one-way shape memory. Beginning with recovery ratio, there were no observable differences between all tested groups. Average recovery ratios were at or above 99% for all groups with standard deviations on the magnitude of 1x10⁻³ %. Fixing ratio followed a similar trend with averages at or above 99% for all groups tested. Additionally, these measurements were just as accurate with standard deviations on the magnitude of 1x10⁻⁴ %. Taking into account the results from each shape memory analysis, it is clear that sterilizing with NOx does not adversely or negatively impact the shape memory behavior of the tBA/BA foams used in this thesis.

In Chapter 4, a preliminary sterility study was conducted to begin quantifying the effectiveness of the NOx sterilization system and working protocol. The study was designed around two different sterility tests that when used in conjunction provide an accurate depiction of the NOx system. Once again foams with 0, 25, and 50% compression ratios were tested to analyze how well the NOx system can sterilize samples with reduced pore sizes. The initial sterility test was done by placing the foams (after they had completed a sterilization cycle) into a test tube filled
with 3 mL of bacterial growth medium. Each test tube was then placed into a shaker at 37°C and 100 RPM so any bacteria present in the sample would have the opportunity to grow. Contamination was then subjectively judged based on color and turbidity changes. Visual examples of contaminated judgements can be seen in Figure 4-1. Samples that were deemed not to be contaminated were then moved to a secondary sterility test to catch any contamination that did not have a concentration large enough to change the color or turbidity of the media in the initial obviousness test. The second sterility test, commonly known as a streak test was completed by taking an aliquot of medium from each vial used in the initial study and plating on an agar plate. Each agar plate was then placed in an incubator for 48 hours to allow for any bacterial colonies to form. The results of each sterility test indicated positive outcomes for the NOx system because none of the treated samples were contaminated. Only the compressed unsterilized samples showed contamination in the initial obviousness test and there were no contaminated samples in the streak test. While this study is far from conclusive, it does indicate the NOx system is effective at sterilizing porous scaffolds even the pore size is dramatically reduced.

5.2 Recommendations for Future Work

5.2.1 NOx System and Protocol Developments

Highlighted in Chapter 2 were general improvements to the NOx system that drastically improved its functionality. These included installing a vacuum pressure gauge, fixing pressure leaks, changing the size of the syringe used to transfer humid air, and generating humidity in a more reproducible way. All of these improvements including the method for locating the source of pressure leaks (soapy water test) are paramount to the effectiveness of the NOx system and should remain a part of the protocols throughout its lifetime. Also highlighted in Chapter 2 are
three specific steps in the protocol that remain underdeveloped and should be the focus of future improvements.

Specifically the problematic steps are the amount of NO added during a sterilization cycle, humidity of the house air, and temperature of the humid air added towards the end of the sterilization cycle. The amount of NO added is measured as a function of the total system pressure and currently the acceptable amount of NO to add is in a very narrow range. Conducting experiments to either widen that range or gather additional evidence that proves 2-3 mmHg is the appropriate amount to add would improve this step. Specifically conducting experiments with tBA/BA foams that have different tBA/BA compositions and therefore different glass transition temperatures would be a great starting point for this type of analysis. Now regarding the humidity of the house air, the largest improvement would be simply to develop a method for accurately measuring or controlling it. This could mean attaching a humidity sensor to the air line, or building an enclosure around the NOx system to limit the exposure of anything not being directly added to the system. Lastly, being able to measure the temperature of the humid air before it is added to the sample chamber would help dramatically because then theoretically the temperature of the air would never exceed the glass transition temperature of the material being sterilized which prevents premature shape recovery.

5.2.2 Shape Memory Analysis

In Chapter 3 the effects of NOx sterilization on the shape memory behavior of a 92/8 wt% tBA/BA foam were analyzed two different ways. Both the automated and manual analysis revealed that NOx does not induce or have a negative effect on the shape memory behavior of the tBA/BA foams. Recommendations for future studies would be to first retain an experimental layout that has an automated and manual analysis because it is important to have a direct check of what is going during sterilization and to have automated measurements for literature comparison. Material
recommendations for future studies in regards to foams would be to vary the composition of tBA/BA and then change the monomers entirely. This would expand the library of knowledge on how NOx effects materials with different mechanical properties. Additionally, it has been mentioned that the primary motivation for this work was evidence that NOx adversely affects the shape memory behavior of a polyurethane SMP sleeve [2]. This material should be revisited to determine if the protocol improvements discussed in Chapter 2 have a statistically significant improvement as compared to the older protocol.

5.2.3 Sterility Analysis

Chapter 4 presented and discussed the layout and results of a two-tiered preliminary sterility study. In this study, an initial obviousness test was followed in conjunction by a streak test to give an accurate representation of the effectiveness of the NOx system and protocol. While the results of the study were positive in that none of the treated samples showed contamination, it was interesting to note that the initial obviousness test was sufficient in identifying all contaminated samples. Therefore recommendations for future work would be to design larger sterility assays. Regardless of the material, larger studies should have technical replicates for every test group and focus primarily on efficiency. As soon as the first samples begin to show contamination in the obviousness test, the streak test should be scheduled for the next day. If no gross contamination is observed after 7 days, the obviousness test can conclude. In regards to the use of the streak test as a backup to the obviousness test, because it did not identify any newly contaminated samples, it is recommended that streak tests do not need to be performed for every independent replicate. To elaborate, conducting streak tests on a smaller portion of samples would provide a qualitative check that the assay is working correctly while not taking the time and materials to show sterility in every single sample not contaminated after the obviousness test. Lastly a strong
recommendation for future work would be conduct studies with other growth mediums as all contaminates will not grow in bacterial medium.

5.3 References


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