Targeted Delivery of Drug Loaded Albumin Nanoparticles to Emphysematous Lungs to Preserve Elastin and Mitigate Matrix Metalloproteinase Activity

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TARGETED DELIVERY OF DRUG LOADED ALBUMIN NANOPARTICLES TO EMPHYSEMATOUS LUNGS TO PRESERVE ELASTIN AND MITIGATE MATRIX METALLOPROTEINASE ACTIVITY

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the Graduate School of
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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in U.S. following cancer and heart disease. COPD is an umbrella term for two chronic pathological conditions, namely chronic bronchitis and emphysema that are seen in patients. According to American Lung Association, 12.7 million Americans have been diagnosed with COPD, while 24 million people have impaired lung function, considered as underdiagnosed for the disease resulting in a huge cost to the nation of about $50 billion. Emphysema is an airway disease in which inflammation mediated elastin damage occurs over a long period. Owing to protease/anti-protease imbalance caused by chronic inflammation, various “elastases” can degrade elastin. Loss of elastin in the lungs has been shown to correlate with loss of lung function in patients. Currently available treatments for COPD aim at only providing temporary relief to the patients by mitigating inflammation or by the action of bronchodilators. Elastin breakdown and chronic inflammatory conditions are hallmark of emphysema. We have developed unique way to deliver nanoparticles tagged with elastin antibody that recognizes degraded elastin in the cardiovascular disease sites. In this research, we have shown that this targeted delivery can be extended to emphysematous lungs to deliver doxycycline and pentagalloyl glucose (PGG) in an attempt to inhibit matrix metalloproteinase (MMP) activity and to preserve elastin in the lung tissue using both in vitro and in vivo approaches.
I would like to dedicate this work to my parents Gayathri and Visweswara Sastry, who have always put my life and comfort before themselves and have always supported me in every walk of my life. If not for their love and support, I will not be standing where I am now.
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# TABLE OF CONTENTS

**ABSTRACT** ........................................................................................................ ii

**ACKNOWLEDGEMENTS** ......................................................................................... iv

**TABLE OF CONTENTS** ............................................................................................ vi

**LIST OF FIGURES** ................................................................................................... xiii

**LIST OF TABLES** ..................................................................................................... xvii

**LIST OF ABBREVIATIONS** ....................................................................................... xviii

1  *Literature review* ...................................................................................................... 1

1.1 The respiratory system ......................................................................................... 1

1.2 Composition of lung tissue ................................................................................... 2

1.3 Lung physiology ...................................................................................................... 2

1.4 Lung volumes .......................................................................................................... 5

1.5 Lung disorders ......................................................................................................... 6

1.6 Symptoms and diagnosis ....................................................................................... 8

1.7 Emphysema: Molecular pathophysiology ............................................................. 9

1.8 Oxidative stress in COPD .................................................................................... 10

1.9 Elastin degradation and its importance .................................................................. 12

1.10 Elastin loss in COPD ........................................................................................... 15
1.11 Elastin degradation as biomarker in COPD 16
1.12 Experimental models for studying emphysema 16
1.13 Treatments 22
1.14 Drug delivery to lungs 26
1.15 Barriers for drug development for COPD 28
1.16 Polyphenols as enzyme inhibitor, anti-oxidant, and elastin stabilizing agents 30

2 Project rationale and specific aims 33

2.1 Innovation of current research 35

3 SPECIFIC AIM 1: To investigate whether elastin-antibody conjugated albumin nanoparticles can be targeted to emphysematous lungs 38

3.1 Introduction 38

3.2 Materials and Methods 39

3.2.1 Preparation of DiR dye loaded BSA nanoparticles 39
3.2.2 Tagging NPs with elastin antibody 40
3.2.3 *In vitro* nanoparticle uptake 41
3.2.4 Targeted delivery of ELN-DiR-BSA NPs *in vivo* 42
3.2.5 Lung compliance measurement 43
3.2.6 DiR signal measurement 43
3.2.7 Histological analysis 43
3.2.8 Statistical analysis

3.3 Results

3.3.1 Nanoparticle uptake

3.3.2 In vivo targeting study

3.3.3 Histology

3.4 Discussion

3.5 Conclusion

4 SPECIFIC AIM 2: To investigate whether targeted nanoparticles can deliver active doxycycline to lungs and inhibit MMP activity

4.1 Introduction

4.2 Materials and Methods

4.2.1 Preparation of Doxycycline loaded BSA nanoparticles (DOX-BSA NPs)

4.2.2 Tagging NPs with elastin antibody

4.2.3 Characterization of doxycycline NPs

4.2.4 Cell culture for cytotoxicity

4.2.5 In vitro cytotoxicity of Doxycycline NPs

4.2.6 Functional drug release from nanoparticles

4.2.7 Targeted delivery of ELN-DOX-BSA NPs in vivo

4.2.8 Measurement of MMP activity in BALF

4.2.9 Immunofluorescence for elastin and MMP-9
5 SPECIFIC AIM 3: To investigate whether PGG treatment can help pulmonary fibroblasts make more elastin while suppressing MMP activity in a cell culture model of emphysema

5.1 Introduction

5.2 Materials and methods
  5.2.1 Preparation of cigarette smoke extract (CSE)
  5.2.2 Pulmonary fibroblast cell culture
5.2.3 FASTIN assay

5.2.4 PGG concentration characterization

5.2.5 Cigarette smoke extract characterization

5.2.6 In vitro emphysema model

5.2.7 mRNA extraction and RT-PCR

5.2.8 Quantitative PCR

5.2.9 Protein isolation

5.2.10 Gelatin zymography and Reverse zymography

5.2.11 Reactive oxygen species (ROS) analysis

5.2.12 Immunofluorescence imaging of elastin

5.3 Results

5.3.1 Pentagalloyl glucose (PGG) concentration characterization

5.3.2 Cigarette smoke extract (CSE) characterization

5.3.3 Relative expression of LOX, LOXL1 and ELN genes

5.3.4 Elastin quantification

5.3.5 LOX activity

5.3.6 Gelatin zymography and Reverse zymography

5.3.7 Immunofluorescence of elastin

5.3.8 ROS analysis

5.4 Discussion

5.5 Conclusion
SPECIFIC AIM 4: To investigate whether PGG loaded nanoparticles can be targeted to emphysematous lungs and whether they increase elastin matrix deposition, thus bringing back the elastic recoil of lungs

6.1 Introduction

6.2 Materials and methods

6.2.1 Ex vivo PGG treatment

6.2.2 Elastase inhibition by PGG

6.2.3 Preparation of DiR dye loaded BSA nanoparticles (DiR-BSA NPs)

6.2.4 Tagging NPs with elastin antibody

6.2.5 In vivo targeting of nanoparticles to mice lungs

6.2.6 Nanoparticle delivery via inhalation

6.2.7 Preparation of PGG loaded BSA NPs

6.2.8 Targeted delivery of PGG-BSA NPs in vivo

6.2.9 Measurement of air volume and mean lung volume intensity

6.2.10 Measurement of lung functional parameters

6.2.11 Measurement of MMP activity

6.2.12 Histology

6.3 Results

6.3.1 Ex vivo PGG treatment

6.3.2 Elastase inhibition by PGG

6.3.3 In vivo targeting of nanoparticles to mice lungs
6.3.4 Histology

6.3.5 Nanoparticle delivery via inhalation

6.3.6 Measurement of air volume and mean lung volume intensity

6.3.7 Measurement of lung functional parameters

6.3.8 Measurement of MMP activity

6.3.9 Histology

6.4 Discussion

6.5 Conclusion

7 Conclusions and recommendations

7.1 Conclusions

7.2 Recommendation for future work

8 References
LIST OF FIGURES

Figure 1-1: Human Respiratory System ................................................................. 1
Figure 1-2: Compliance diagram for lungs ............................................................... 4
Figure 1-3: Graphical demonstration of chronic bronchitis and emphysema, the two pathological conditions of COPD ................................................................. 7
Figure 1-4: Schematic view of pathophysiological mechanism of emphysema..... 9
Figure 1-5: Anti-oxidant strategies based research in COPD ............................... 11
Figure 1-6: TEM structure of elastic fiber .............................................................. 12
Figure 1-7: Schematic representation of elastin synthesis and assembly by cells 14
Figure 1-8: Effects of cigarette smoke on lung cells .............................................. 19
Figure 1-9: Chemical structure of PGG .................................................................. 31
Figure 3-1: Schematic diagram showing conjugation of elastin antibody to DiR-BSA NPs. .................................................................................................................. 40
Figure 3-2: Timeline graph of study involving targeted delivery of DiR-BSA NPs in rats .................................................................................................................. 42
Figure 3-3: Nanoparticle uptake of DiR-BSA NPs ............................................... 45
Figure 3-4: NP targeting to elastase damaged lungs .......................................... 46
Figure 3-5: Bar chart showing biodistribution of NPs ............................................ 47
Figure 3-6: Histological examination of saline and elastase group rat lungs. ..... 48
Figure 3-7: Fluorescence images of ELN-DiR-BSA NPs ................................. 49
Figure 4-1: Timeline graph for animal study for targeted delivery of DOX-BSA NPs in rats .................................................................................................................. 59
Figure 4-2: Scanning electron microscopy images of DOX-BSA NPs. .......................... 64
Figure 4-3: Release curve of doxycycline from DOX-BSA NPs .............................. 65
Figure 4-4: In vitro cytotoxicity and nanoparticle uptake by cells. ......................... 66
Figure 4-5: Bar chart showing MMP activity suppression by doxycycline released from nanoparticles ........................................................................................................ 67
Figure 4-6: MMP activity in the BALF measured using specific FRET substrates for MMPs 2, 9 and 12 .................................................................................................................................................................... 67
Figure 4-7: Immunofluorescence assay for MMP 9 ............................................ 68
Figure 4-8: In situ zymography of frozen lung sections ...................................... 69
Figure 4-9: Casein zymography images showing MMP-12 activity .................... 70
Figure 5-1: Live/Dead assay of rat pulmonary fibroblasts grown for 1 week in different concentrations of PGG dissolved in DMSO ......................................................... 82
Figure 5-2: Bar graph showing quantified matrix elastin after three weeks, from the cell culture of rat pulmonary fibroblasts grown in different PGG concentrations 83
Figure 5-3: Viability of cells grown in various concentrations of CSE ............... 84
Figure 5-4: Bar graphs showing relative expression of LOX and elastin genes... 86
Figure 5-5: Bar charts showing elastin quantified ............................................. 87
Figure 5-6: Bar charts showing LOX activity quantified in rat pulmonary fibroblasts ............................................................................................................................................... 88
Figure 5-7: Gelatin zymography images showing MMP 9 activity in cell culture medium ...................................................................................................................................................... 89
Figure 5-8: Reverse zymography images showing TIMP activity in cell culture medium .......................................................................................................................... 90

Figure 5-9: Immunofluorescence imaging of cell cultures at 3 weeks to visualize elastin ........................................................................................................................................ 91

Figure 5-10: Visualization of reactive oxygen species (ROS) in rat pulmonary fibroblasts .......................................................................................................................... 92

Figure 6-1: Nebulizer and pie cage set up for inhalation delivery of solutions. 103

Figure 6-2: Timeline graph of animal study to deliver DiR-BSA NPs via inhalation and intravenous injections in mice ...................................................................................... 103

Figure 6-3: Timeline graph of animal study to deliver PGG-BSA NPs in mice. 105

Figure 6-4: Lung segmentation and mean lung volume intensity measurement using CT-An software. ................................................................................................................. 106

Figure 6-5: Results showing protection of elastin by PGG from elastase challenge ex vivo. ...................................................................................................................................... 109

Figure 6-6: Bar chart showing elastin quantified in control and PGG fixed lung samples following elastase challenge. ................................................................. 110

Figure 6-7: Line graph showing inhibition of elastase activity by various concentration of PGG ...................................................................................................................... 111

Figure 6-8: Fluorescence from DiR NPs show successful targeting of anti-elastin antibody tagged nanoparticles to only lungs with elastin damage ....................... 112

Figure 6-9: Plot of elastin quantified in control, PPE1 and PPE2 group mice lungs versus DiR fluorescent signal found in them ........................................................................ 113
Figure 6-10: Histological analysis of lung sections from control, PPE1 and PPE2 groups.................................................................................................................................................. 115

Figure 6-11: Fluorescence images of lungs showing nanoparticle targeting via different routes ........................................................................................................................................... 116

Figure 6-12: Bar charts showing lung volume parameters measured using micro CT .................................................................................................................................................. 117

Figure 6-13: Bar chart showing dynamic lung compliance (Cdyn) values ........ 118

Figure 6-14: Bar chart showing lung resistance values in all groups of mice. .... 119

Figure 6-15: Bar chart showing tidal volume values in all groups of mice....... 119

Figure 6-16: Bar charts showing MMP 2&9 and 12 activities quantified in lung samples.................................................................................................................................................. 120

Figure 6-17: Histological examination of mice lungs........................................ 122
LIST OF TABLES

Table 1-1. Lung volumes and their average values in humans............................ 6
Table 1-2. GOLD Guidelines for classification of air flow limitation in COPD ... 9
Table 1-3. Summary of CSE effects in various cell culture models................. 18
Table 4-1. Characterization of doxycycline loaded bovine serum albumin nanoparticles. ........................................................................................................... 55
Table 4-2 Properties of DOX-BSA NPs .......................................................... 65
Table 5-1. Primer sequences and amplicon sizes for genes of interest............ 78
LIST OF ABBREVIATIONS

1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide (DiR)
Abdominal aortic aneurysm (AAA)
Alpha-1 anti-trypsin (AAT)
Bovine serum albumin (BSA)
Bronchoalveolar lavage fluid (BALF)
Chronic obstructive pulmonary disease (COPD)
Cigarette smoke extract (CSE)
Dimethyl sulfoxide (DMSO)
Elastin binding protein (EBP)
Epigallocatechingallate (EGCG)
Expiratory reserve volume (ERV)
Extracellular matrix (ECM)
Forced expiratory volume (FEV1)
Forced vital capacity (FVC)
Functional residual capacity (FRC)
Hemotoxylin and eosin (H&E)
Human vascular endothelial cells (HuVECs)
Inspiratory capacity (IC)
Inspiratory reserve volume (IRV)
Interleukin (IL)
Latent TGF-β binding protein (LTBP)
Leukotriene B4 (LTB4)
Lipopolysaccharide (LPS)
Long acting bronchodilators (LABAs)
Long acting muscarinic antagonists (LAMAs)
Lysyl oxidase (LOX)
Metalloproteinases (MMPs)
Microfibril associated glycoproteins (MAGPs)
Mitogen-activated protein (MAP)
Nanoparticles (NPs)
Pentagalloyl glucose (PGG)
Phosphate buffered saline (PBS)
Phosphodiesterase 4 (PDE4)
Poly (lactic acid) (PLA)
Poly ethylene glycol (PEG)
Polyoxalate (HPOX)
Porcine pancreatic elastase (PPE)
Reactive oxygen species (ROS)
Residual volume (RV)
Reverse transcriptase polymerase chain reaction (RT-PCR)
Smooth muscles cells (SMCs)
Tidal volume (TV)
Tissue growth factor-beta1 (TGF-β1)
Tissue inhibitor of metalloproteinases (TIMPs)
Total lung capacity (TLC)
Tumor necrosis factor-α (TNF- α)
Verhoeff’s van gieson (VVG)
Vital capacity (VC)
1 LITERATURE REVIEW

1.1 The respiratory system

Human respiratory system (Figure 1-1) is a fascinating blend of organs that facilitates one of the most important functions of the body, respiration. Anatomically, it is broadly divided into upper and lower respiratory tracts. Upper respiratory tract consists of nasal cavity, pharynx, and larynx while the lower respiratory tract forms the crux of respiratory system where the exchange of gases happens. Trachea, bronchi, and lungs form the lower respiratory tract. Gross anatomy of lungs shows us that the trachea splits into two large bronchi (right and left) which further divide into 23 generations of air ducts, the end

Figure 1-1: Human Respiratory System (http://www.6aming.com/private/human-respiratory-system-diagram-labeled/attachment/diagram-of-human-respiratory-system-health-pinterest-inside-human-respiratory-system-diagram-labeled/)
of which forms the smallest functional units of lungs, called alveoli [2]. Alveoli interface with the surrounding capillaries to allow oxygen and carbon dioxide exchange.

1.2 Composition of lung tissue

Lungs are composed of cellular, vascular and extracellular tissue components. Epithelial cells provide continuous lining throughout the respiratory system. At the distal end of respiratory bronchioles, where they form alveoli, two types of alveolar cells (type I and II) start to appear. Type I cells are squamous epithelium, which provide thinnest blood-air interface for exchange of gases, while type II cells form a part of alveolar septa and generate surfactant fluid. They also differentiate into type I cells to replenish them, which lack the ability to divide [3]. Alveolar macrophages form an important part of lungs’ cellular network, with a vital immunological role to play in clearance mechanism and also in pathological responses [4].

Vasculature of lungs consists of bronchial and pulmonary circulations. Pulmonary circulation consists of pulmonary arteries bringing in the deoxygenated blood, which profusely divides into capillaries for gas exchange. Pulmonary veins transport back the oxygenated blood to the systemic circulation. Bronchial circulation intertwines with pulmonary circulation in a contrasting hemodynamic manner to supply blood to the lung tissue [5].

1.3 Lung physiology

The lungs work to refresh the oxygen supply to the body. The left lung has two lobes owing to the presence of heart on left side while the right lung has three lobes.
Trachea divides into bronchi, which in turn divide into bronchioles and finally end up as alveoli. Alveoli are the places where gas exchange occurs from capillaries. On an average, our lungs have about half a liter of blood at any given point of time. The negative pressure acting on the alveoli makes sure no blood seeps into them except for very small amounts of fluid, which is safely taken back by the lymphatic system. The blood-air barrier at the point where capillaries pass alveoli forms the thinnest possible surface for gas diffusion into and out of capillaries.

The lungs do this job of getting fresh oxygen in and spent carbon dioxide out by the process of respiration [5-10]. Respiration essentially consists of two tasks i.e. inspiration and expiration. The physiology of working of lungs involves its expansion and collapse during these tasks respectively. Primarily the diaphragm contracts to pull the lungs downwards and expand them. It relaxes to allow the lungs to contract. Secondary way of expanding and contracting lungs involves raising and lowering of the ribs involving intercostal muscles, scaleni, sternocleidomastoid, anterior serrati and abdominal recti muscles.

The pressure of liquid in pleural space, called pleural pressure, is about -5 cm of water, which holds the lungs open at rest. The pressure inside alveoli, called alveolar pressure, remains very close to zero. During inspiration, the alveolar pressure falls slightly below atmospheric pressure and the pleural pressure goes down to -7.5 cm of water. This gives enough suction to draw 500 ml of fresh air. During expiration, the values go back in the same manner. The difference between alveolar and pleural pressures is called
transpulmonary pressure. The extent to which the lungs will expand for a unit increase in transpulmonary pressure is called the lung compliance. Lung compliance is the volume change that could be achieved in the lungs per unit pressure change. On an average the total compliance of both our lungs is about 200 ml of air per cm of water of transpulmonary pressure. The plot of pressure changes versus lung volume changes is called a compliance diagram and looks like a hysteresis curve (Figure 1-2).

The compliance of the lungs comes from two factors i.e. (1) elastic forces of lung tissue and (2) elastic forces caused by surface tension of surfactant that lines inner walls of alveoli. Elastic forces in lung tissue come from mainly elastin and collagen network present in the interstitium. During inspiration, the elastic fibers are extended and owing to their action of hydrophobic interactions they tend to collapse thus pulling the lungs inwards and expelling the air. On the other hand, the surface tension of the fluid in alveoli tends to collapse the alveoli thereby creating a positive pressure to expel the air out. A type of
protein called surfactant protein present in this fluid reduces its surface tension. This helps in creating a much less positive pressure with which the lungs expel the air, so that the inspiration doesn’t become a hard task. The pressure with which the alveoli expel the air can be calculated using the formula below.

\[
\text{Pressure} = \frac{2 \times \text{Surface tension}}{\text{Radius of alveolus}}
\]

1.4 Lung volumes

To know more about the functioning of lung, certain volumes can be measured at various points of respiration. There are four pulmonary volumes. Various capacities of lungs are defined as sum of two or more of the pulmonary volumes. These are listed in Table 1-1.

A very good understanding of the functioning of normal lungs is important to appreciate the pathobiology of chronic obstructive pulmonary disease (COPD). In clinical settings, these parameters are used to assess the severity of the disease in a patient. In research involving animal models, evaluating the compliance, lung volumes, and capacities forms an important part of the experiment if investigating lung regeneration.
Table 1-1. Lung volumes and their average values in humans

<table>
<thead>
<tr>
<th>Volume</th>
<th>Definition</th>
<th>Value (ml of air)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tidal volume (VT)</td>
<td>Volume of air inspired or expired with normal breathing</td>
<td>500</td>
</tr>
<tr>
<td>2. Inspiratory Reserve</td>
<td>Volume of air that can be inspired after tidal volume with full force.</td>
<td>3000</td>
</tr>
<tr>
<td>Volume (IRV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Expiratory Reserve Volume</td>
<td>Volume of air that can be expired forcefully apart from tidal volume</td>
<td>1100</td>
</tr>
<tr>
<td>(ERV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Residual volume (RV)</td>
<td>Volume of air that remains in the lungs after forceful expiration</td>
<td>1200</td>
</tr>
<tr>
<td>5. Inspiratory capacity</td>
<td>Maximum amount of air a person can breathe after expiration</td>
<td>3500</td>
</tr>
<tr>
<td>(IC)</td>
<td>IC = VT + IRV</td>
<td></td>
</tr>
<tr>
<td>6. Functional Residual</td>
<td>Amount of air that remains in the lungs after a normal expiration</td>
<td>2300</td>
</tr>
<tr>
<td>Capacity (FRC)</td>
<td>FRC = ERV + RV</td>
<td></td>
</tr>
<tr>
<td>7. Vital capacity (VC)</td>
<td>Amount of air that a person can expel following a maximum inspiration and maximum expiration</td>
<td>4300</td>
</tr>
<tr>
<td>8. Total Lung Capacity (TLC)</td>
<td>Maximum volume to which the lungs can be expanded. TLC = IRV + VT + ERV + RV</td>
<td>5800</td>
</tr>
</tbody>
</table>

1.5 Lung disorders

Chronic pulmonary disorders are broadly classified into restrictive and obstructive disorders based on how the lung function is hindered. In restrictive disorders like idiopathic pulmonary fibrosis, sarcoidosis, silicosis etc., patients have a trouble in inhaling to the full lung capacity. Their ability to fully expanding their lungs is restricted because of the stiffened interstitium. On the other hand, obstructive lung diseases like chronic obstructive pulmonary disease and asthma cause patients to exhale with difficulty because of the degraded extracellular matrix, which causes the airways to collapse [11].
Chronic obstructive pulmonary disease is the third leading cause of death in U.S. following cancer and heart disease. In the year 2009, the number of people who died because of COPD is estimated to be about 134,000. According to the data published on “Trends in COPD” by American Lung Association in 2013, 12.7 million Americans have been diagnosed with COPD while a whopping 24 million people have impaired lung function, considered as underdiagnosed for the disease [12]. The devastating effect of the disease can not only be felt in the mortality rates but also in the projected economic burden. In 2010 it was projected approximately that the cost to the nation was $49.9 billion for COPD [12].

![Figure 1-3: Graphical demonstration of chronic bronchitis and emphysema, the two pathological conditions of COPD. (http://www.livingwellwithcopd.com/en/what-is-copd.html).](http://www.livingwellwithcopd.com/en/what-is-copd.html)

According to the Global initiative for COPD, this disease is characterized by airflow limitation along with a range of pathological changes in the lung. COPD is an umbrella term encompassing two main pathological conditions chronic bronchitis and emphysema (Figure 1-3). These extreme conditions of COPD can exist either
independently or together in a patient. In COPD associated condition emphysema, lung compliance is severely affected due to the irreversible damage that happens to the elasticity of lungs. Thus, they become less elastic and their compliance increases. Although COPD has been majorly linked to smoking as one of its major causative factors, recent advancements in research has made it possible to link genetic predisposition for the development of clinically significant disease [13-16]. The major genetic risk factor associated is the alpha-1 anti-trypsin (AAT) deficiency, which is a protease inhibitor of elastase. AAT deficiency is a rare recessive disorder in which patients have decreased levels of AAT [17]. Exposure to fine particulate matter in work environments and air pollution are considered other minor causes of emphysema [18, 19].

1.6 Symptoms and diagnosis

Clinical symptoms of COPD include dyspnea, wheezing, cough and decreased expiration capacity. The volume of air that is expelled out at the end of exhalation is called Forced Vital Capacity (FVC) and the volume of air that is expelled in one second after complete inhalation is called Forced Expiratory Volume (FEV1). The ratio FEV1/FVC gives an estimate of the presence and severity of the disease, Table 1-2 [20-22].
Table 1-2- GOLD Guidelines for classification of air flow limitation in COPD

<table>
<thead>
<tr>
<th>GOLD 1</th>
<th>Mild</th>
<th>FEV1 &gt; 80% predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD 2</td>
<td>Moderate</td>
<td>50% &lt; FEV1 &lt; 80% predicted</td>
</tr>
<tr>
<td>GOLD 3</td>
<td>Severe</td>
<td>30% &lt; FEV1 &lt; 50% predicted</td>
</tr>
<tr>
<td>GOLD 4</td>
<td>Very Severe</td>
<td>FEV1 &lt; 30% predicted</td>
</tr>
</tbody>
</table>

1.7 Emphysema: Molecular pathophysiology

Currently accepted hypothesis about emphysema is that chronic exposure to cigarette smoke, particulate matter causes irritation in the alveoli, and this triggers an

![Diagram showing pathophysiological mechanism of emphysema](image)
inflammatory response. This response attracts many types of inflammatory cells like macrophages, neutrophils and lymphocytes to the area. Cytokines, reactive oxygen species, prostaglandins, leukotrienes, proteases etc. released by these cells mediate the progression of the disease. This leads to a protease/anti-protease imbalance in the lungs, which causes the breakdown of elastin [23]. The pathology of emphysema consists of other changes. For example, lung fibroblasts change their phenotype [24], degradation products of elastin fibers act as chemoattractants to inflammatory cells [25], the inflammatory response shifts to chronic phase, alveoli tend to enlarge, small air ways constrict allowing less air to escape during exhalation and finally the irreparable loss of elastin network leaves macroscopically visible holes in the lungs [26]. Elastin in the lung is degraded by enzymes called “elastases” [27, 28]. A few members of serine (e.g. neutrophil elastase, pancreatic elastase), cysteine (Cathepsin K) and metalloproteinases (MMP-2, MMP-9 and MMP-12) can cleave elastin. In diseases like abdominal aortic aneurysms (AAA) and COPD factors like inflammation and oxidative damage initiate and aggravate the elastin degradation [29]. Elastin derived peptides can act as chemo-attractants to the inflammatory cells, and the vicious cycle of elastin degradation and disease progression continues (Figure 1-4).

1.8 Oxidative stress in COPD

Oxidative stress has been shown to exacerbate COPD [30]. Oxidative species like superoxide radical, hydrogen peroxide, carbonyl stress markers like nitrotyrosine and lipid peroxidation products like 8-isoprostane, 4-hydroxy 2-nonenal and malondialdehyde (MDA) have been found in COPD [31-33]. Intrinsic anti-oxidants like glutathione, ascorbic acid, tocopherol and uric acid in the lung try to keep these levels balanced. But in COPD
patients, carbonylation and nitration reduce the activity and expression of an important transcriptional co-repressor histone deacetylase 2 (HDAC2), which is essential for the suppression of activated inflammatory genes and the anti-inflammatory actions of corticosteroids [34, 35]. This contributes to the inflammation in airways in COPD [54 55]. It has also been found that increased oxidative stress can lead to the release of chemokines that can attract inflammatory cells like dendritic cells, monocytes, and lymphocytes, which in turn elevate the levels of interleukins IL-17 and -18 [36] [37]. Several therapies have been studied to combat oxidative stress (Figure 1-5).

![Figure 1-5: Anti-oxidant strategies based research in COPD [1].](image)
1.9 Elastin degradation and its importance

Elastin is a remarkable protein present in the extracellular matrix (ECM) of our tissues. Its ability to render elastic properties to some vital tissues like arteries, skin and lungs marks the physiological importance of this fibrous protein. With a half-life period of 74 years, majority of elastin deposition occurs during embryonic and childhood stages [38, 39]. Elastin synthesis has been documented in fibroblasts, smooth muscle cells, chondrocytes and endothelial cells [40-43]. While most of the vertebrate aortic tissue consists of up to 50% of elastin, the amount of elastin in lungs differs across species. It can range from as low as 2% in rodents to about 28% in humans [44]. Elastin in the lungs appear as concentric circles in pulmonary arteries. As they reach the lowest level of organization, they appear as fine mesh in alveolar walls.

Elastin is made up of elastic fibers that have two major components, one amorphous and one fibrillar (Figure 1-6). The extensively cross-linked elastin of the amorphous component comprises 90% of the fiber. The fibrillar component, is a collection of microfibrils, that are rich in acidic glycoproteins and is about 8-16 nm long.

Figure 1-6: TEM structure of elastic fiber
Many different proteins like fibrillins, microfibril associated glycoproteins-1 and 2 (MAGPs-1 and -2), latent TGF-β binding protein (LTBP) and fibulins contribute the anchoring of elastin and provide it with a structural coating [46]. Fibrillin-1 largely makes up microfibrils in ECM that likely anchor the forming elastic fibers [47]. Lysyl oxidases (LOX) are a group of five enzymes including LOX, and LOXL 1-4, which cross-link tropoelastin and aid the process [48].

Elastin primary structure is mainly composed of hydrophobic amino acids like glycine and proline [49]. Characteristic peptide sequence repeats VPGVG and PGVGV are seen in the elastin protein [50]. Tropoelastin, is a 72kDa protein that is secreted from cells [51]. It has been shown in mice that elastin expression reaches the highest during postnatal days 0 to 14 and then decreases rapidly to remain likewise throughout the life [52]. Genetic mutations in the elastin gene have been shown to contribute to susceptibility of lung disease [53].

Initially, tropoelastin gene is expressed by fibroblasts or vascular smooth muscle cells and then tropoelastin molecules are secreted into the ECM [54]. The tropoelastin molecules begin to accumulate on the surface on the cell to create 1-micron spherules. This accumulation of tropoelastin aids coacervation of tropoelastin molecules. Eventually the tropoelastin is oxidized by the lysyl oxidase enzymes and followed by aldol condensation and Schiff base reactions among lysyl residues to crosslink elastic fibers with desmosine and isodesmosine links. The microfibrils that exist in the ECM are transported to where the new elastin is being formed, by members of the fibulin protein family. The elastic fiber
produced as the final product is very stable and able to give human tissue the ability to stretch and recoil [53]. Smooth muscles cells (SMCs) and fetal lung fibroblasts go through a different process of binding to tropoelastin. SMCs, much like chondrocytes, use heparin sulfate chains through proteoglycans on the cells surface to bind to the tropoelastin. In the fetal lung, fibroblasts use heparan sulfate moiety and integrin αvβ3 receptors [55].

Elastin binding protein (EBP), made of a 67-kDa peripheral subunit attached to two membrane bound proteins of 61 and 55 kDa, carries tropoelastin molecules to fiber formation sites on the surface of cells. Initially, tropoelastin binds to an EBP complex that is intact. The 67-kDa subunit loses its affinity for tropoelastin and the membrane bound

Figure 1-7: Schematic representation of elastin synthesis and assembly by cells
protein when a sugar moiety binds to the EBP. The loss of affinity leads to tropoelastin being released onto growing elastic fibers [45]. The process of elastic fiber assembly is shown schematically in Figure 1-7.

### 1.10 Elastin loss in COPD

To perform the respiration task effectively, lungs need to be compliant enough. The compliance of the lungs comes from two factors, namely (1) elastic forces of lung tissue and (2) elastic forces caused by surface tension of surfactant that lines the inner walls of alveoli. Elastic forces in lung tissue come from mainly elastin and collagen network present in the interstitium. During inspiration, the elastic fibers are extended and owing to their elastic action they tend to collapse during exhalation, thus pulling the lungs inwards and expelling the air. In COPD associated condition emphysema, the irreversible damage that happens to elastin protein in lungs severely affects lung compliance. Loss of this elastin network causes alveoli to enlarge and decrease gas exchange capacity. Physiologically, the lungs lose their elastic recoil, which cannot be reversed fully.

As we age, elastin gene expression decreases dramatically leading to no elastin production, so the tissues must rely on the elastin deposition that occurs in the womb and early in life [56]. In order to make elastin, tropoelastin molecules must interact and then crosslink, forming insoluble elastic fiber. The vital role that elastin degradation plays in the development and progression of COPD has been studied in many animal models [57, 58].
1.11 Elastin degradation as biomarker in COPD

Elastin fibers have very little turnover. Degradation of such elastic fibers is seen in emphysema. As a result, elastin damage is used as a marker for this disease. It has been shown in COPD patients that less elastin is present in the distal lung parenchyma and small airways [59, 60]. Merrilees et al., have observed a positive correlation between elastin fiber fraction in the lungs and forced expiratory volume (FEV-1) [61]. Elastin degradation results in elastin derived peptides (EDPs), which have been shown to be chemoattractants for monocytes. Sellami et al., have even shown that intra-tracheal injection of VGVAPG amino acid (one of the EDP sequences) causes emphysema in a murine model [62]. He et al., have characterized about 40 peptides by digesting human lung samples with MMP-12 and HNE and compared thus derived peptides with those detected in patients [63]. They have observed some of these peptides circulating in COPD patients while these were completely absent in normal subjects.

1.12 Experimental models for studying emphysema

Experimental models of COPD have helped us understand many aspects of pathophysiology of the disease as well as therapeutics’ effect on lungs. Models used for studying COPD can mainly be divided as in vitro, in vivo, and in silico models. Researchers have tried looking at the effect of various individual and combined stimulations on distinct type of cells that participate in the disease pathway by using in vitro models. On the other hand, animal models provided very powerful and insightful details on topics like pathophysiology of COPD and advancement of possible pharmacological treatments. In
silico models have looked at drug delivery simulations and identifying variables that can assist in diagnosing the disease better.

In vitro models: In vitro models of COPD employ a chosen cell type to study the effects of one or more causative agents of the disease [64]. Culture of cells explanted from patients or immortalized cell lines have been used. Exposure of these cells to the inflammatory inputs have helped in understanding certain cellular mechanisms, which would not have been possible in a complex environment such as human disease or animal models of disease.

Out of the known causes of COPD, chronic smoking is strongly linked to the decrease in forced expiratory volume of patients. Naturally, cigarette smoke extract (CSE) has been adopted mostly as a way of inducing inflammatory environment in cell cultures. CSE is nothing but a liquid (either cell culture medium or buffered saline) containing soluble components of cigarette smoke, obtained by bubbling the smoke directly into it. Such solution is then sterilized and added to cell cultures at desired concentrations. With a number of variables governing the preparation of CSE like the cigarettes used, medium for bubbling and type of cells to test CSE with, there appear many ways of utilizing this in vitro stimulant for studying COPD.

Nyunoya et al., have observed that single cigarette smoke extract dose inhibits lung fibroblast proliferation, while multiple exposures to cigarette smoke move cells into an irreversible state of senescence [65]. Nakamura et al., [66] have observed that treatment of human fetal lung fibroblasts with CSE inhibited proliferation and migration of the cells.
Hoshino et al., [67] have also observed similar derogatory effects of CSE on viability of A549 cell line, causing DNA fragmentation and increase in oxidative stress. They have also shed light on the protective effect of N-acetylcysteine (NAC) against CSE exposure. An earlier interesting finding by Laurent et al., shows that cigarette smoke extract blocks the crosslinking of tropoelastin molecules by inhibiting the function of lysyl oxidase [68]. Some of the findings from the literature are presented in Table 1-3.

Table 1-3. Summary of CSE effects in various cell culture models.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Effect of CSE</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>↑Apoptosis</td>
<td>[69]</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>↑Apoptosis, Oxidative stress↑</td>
<td>[70]</td>
</tr>
<tr>
<td>Epithelial</td>
<td>↓Cell viability, Oxidative stress↑</td>
<td>[67]</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>↓ Cell proliferation and migration</td>
<td>[66]</td>
</tr>
<tr>
<td>Epithelial</td>
<td>↑ Epithelial to mesenchymal transition</td>
<td>[71]</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>↑ Proliferation via PKC-PDGFB signaling</td>
<td>[72]</td>
</tr>
<tr>
<td>Endothelial</td>
<td>↑Cell injury via JNK pathway</td>
<td>[73]</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>↑Cellular senescence, ↓ Cell proliferation</td>
<td>[65]</td>
</tr>
<tr>
<td>Endothelial, Platelet</td>
<td>↓ Cellular viability, ↑Aggregation potential</td>
<td>[74]</td>
</tr>
<tr>
<td>T cells, Epithelial</td>
<td>↓ Cytotoxic activity of T cells, ↑IL-8 and MUC5AC</td>
<td>[75]</td>
</tr>
<tr>
<td>Dendritic</td>
<td>↑PGE2 production, ↓DC-induced T-cell proliferation</td>
<td>[76]</td>
</tr>
<tr>
<td>Bronchial epithelial</td>
<td>↓ Chloride secretion, ↑Mucus secretion</td>
<td>[77]</td>
</tr>
<tr>
<td>Dermal fibroblast</td>
<td>↓Cell viability, Oxidative stress↑</td>
<td>[78]</td>
</tr>
<tr>
<td>Alveolar macrophage</td>
<td>↑Apoptosis</td>
<td>[79]</td>
</tr>
<tr>
<td>Bronchial epithelial</td>
<td>↑DNA damage</td>
<td>[80]</td>
</tr>
</tbody>
</table>
Other *in vitro* models include exposure of cells to non-tobacco irritants like fine particulate matter. Li et al., have previously observed that ultrafine particle exposure increases the permeability of epithelial cells using A549 cell line [81]. They have also observed decreasing glutathione levels, which acts as an antioxidant in the body. Lee et al., [82] have experimented with coal dust exposure on type II pneumocytes, which resulted in increased extracellular matrix proteins, regulated by tumor necrosis factor-α (TNF-α) and tissue growth factor-beta1 (TGF-β1). How cigarette smoke can act on lung cells is depicted in Figure 1-8.

![Figure 1-8: Effects of cigarette smoke on lung cells](image)

**In vivo models:** Animal models have contributed greatly towards the advancement of knowledge in COPD. There are many methods mentioned in literature that are used to induce COPD like changes in animal lungs [83-85]. Broadly, animal models of COPD can
be divided as cigarette smoke exposure, elastase instillation and genetic manipulation. Mice are the most commonly used animals for inducing COPD while rats are considered less susceptible to develop COPD[86].

*Cigarette exposure:* Exposure of animals to cigarette smoke is a classical model used by researchers owing to the fact that tobacco smoke is the most important risk factor of COPD. The advantages of such exposure include emphysematous changes to lungs, recruitment of inflammatory cells, and airway fibrosis similar to seen clinically in smokers [87-89]. On the other hand, the exposure model usually requires long time to observe significant changes in the lung. Since there is no set standard on the type of cigarettes, dosage of cigarette smoke given to animals, and delivery systems used in this model, many studies have used their own methods [90-92]. Multitude of parameters such as, importance of inflammatory cells in progression of the disease and possible anti-inflammatory therapies have been studied in detail in mice, rats, guinea pigs, and dogs [93-99].

*Elastase model:* Elastases are enzymes that degrade elastin in the body. Thus it is no surprise that instillation of elastase emerged as one of the widely used and characterized models of emphysema in the animal setting. Porcine pancreatic elastase has been very popular agent used to damage lung structure in animals. The ease of use, quicker damage, and better optimization form the major advantages of the elastase model [91, 100]. However, the disadvantage of the elastase comes from its inability to comprehensively reproduce pathogenesis as seen in clinical findings [101]. Clearance of enzyme from lungs also dictates the extent of damage [102]. Studies reported in the literature differ in the
amount of elastase concentration used to damage the lungs of animals (rats, mice or hamsters), the amount of time allowed for damage and also the intervention of exacerbations like exercise and infections [103-108]. Most common observations of elastase challenge to lungs are enlargement of airspaces, inflammatory cell influx into the lungs, and increased lung compliance[109-112].

**Genetic manipulation models:** Aptly called as second generation models of COPD, genetically manipulated animals have been used to evaluate many key players in its pathogenesis [113]. Pallid mice, which have alpha-1 antitrypsin deficiency, are shown to develop a milder form of emphysema [114]. Beige mice also develop COPD-like lesions due to a defect in neutrophil granules [115]. Genetic models have also been useful to emphasize the importance of MMPs in the pathophysiology of emphysema. D’Armiento et al., have shown that expression of human collagenase gene in transgenic mice leads to alveolar wall disruption with no signs of inflammation or fibrosis [116]. Hautamaki et al., have used macrophage elastase (MMP-12) knock out mice in a cigarette smoke exposure model of emphysema. Their observations lead to the finding that mice lacking MMP-12 fail to develop emphysema. [117].

Targeted mutagenesis has allowed to knock out specific proteins to evaluate their function in disease pathogenesis [57, 58, 118, 119]. Rangasamy et al. showed that disruption of the *Nrf2* gene led to earlier onset and more extensive features of emphysema in mice exposed to cigarette smoke, stressing the importance of this gene [120]. Leco et
al., have observed spontaneous air space enlargement and impaired lung function when tissue inhibitor of metalloproteinases-3 (TIMP-3) has been knocked out [121].

Other animal models have employed a completely different variety of substances to achieve COPD like pathology in the lungs. These include instillation of nitrogen dioxide [122], lipopolysaccharide [123, 124] [125], cigarette smoke extract[126, 127], elastin fragments[128], cadmium[129], copper deficiency[130].

1.13 Treatments

Currently, there is no clear-cut treatment plan available for COPD. There are drugs available to provide temporary relief to patients and to treat the exacerbation of the disease. However, a long-term reliable treatment has not been found for COPD. Treatment of COPD with drugs used for asthma seems inappropriate, as the difference in inflammation is markedly different in both cases. Along with pharmacological treatments, smoking cessation is also advised in patients to curb the disease progression [131]. Pharmacological therapeutics for COPD treatment are divided based upon their course of action. Bronchodilators, mediator antagonists, anti-inflammatory agents and remodeling agents.

*Bronchodilators*: Bronchodilators aim at providing relief to patients by relaxing muscles around airways and by clearing mucus for easier breathing. Depending on the time of their action, there are short, long and ultra-long acting bronchodilators (LABAs and ultra-LABAs), which can have effect up to 24 hours. Carmoteral, indacatero, vilanterol are some of the examples of available bronchodilators [132]. Muscarinic antagonists are drugs that block activity of muscarinic responsive acetylcholine receptor. Acetylcholine
directly acts at muscarinic receptors on airway smooth muscle to cause bronchoconstriction [133]. Long acting muscarinic antagonists (LAMAs) like ipratropium bromide, tiotropium bromide [134], aclidinium bromide [135] have made their advance into the clinical treatment. Tiotropium bromide has been shown to be effective because of its anti-inflammatory properties [136, 137].

Anti-Inflammatory drugs: The use of anti-inflammatory drugs is also a viable therapy as inflammation causes disease progression. Since corticosteroids have proven to be ineffective in reducing inflammation in COPD, new drugs like phosphodiesterase 4 (PDE) inhibitors, TNF-α inhibitors, NF-kB inhibitors, p38 mitogen-activated protein (MAP) inhibitors, adhesion molecule inhibitors, PI-3 K inhibitors, anti-inflammatory cytokines, leukotriene B4 inhibitors, and chemokine inhibitors are being evaluated for their anti-inflammatory efficacies [138-142]. Since human neutrophils are largely involved in COPD, drugs trying to stop the recruitment of neutrophils have surfaced as a way of treatment. Leukotriene B4 (LTB4) is a potent chemoattractant of neutrophils and is increased in COPD patients [143]. In one study, mice lungs were challenged with lipopolysaccharide (LPS) and cigarette smoke solution and treated with inhalable powder of a flower Lonicera japonica. This treatment has shown to decrease TNF-α and IL-6 levels in bronchoalveolar fluid and the number of inflammatory cells in peripheral blood. In addition, authors have also reported that this treatment has induced recovery of elastin and collagen distribution, reduction of caspase-3 expression in lung tissues of COPD mice [144].
**MMP Inhibitors:** Another approach to preserve elastin is to inhibit matrix metalloproteinases that are involved in degradation of elastin protein [145]. In a guinea pig study involving cigarette smoke exposure an MMP9/12 inhibitor AZ11557272 has ameliorated the emphysema conditions [146]. In a cigarette smoke exposure model of emphysema, mice treated with nebulized Ilomastat (MMP inhibitor) have been shown to have reduced macrophage levels, recruitment of neutrophils and also a reduction in air space enlargement [147]. In another study using a broad spectrum MMP inhibitor CP-471,474, delayed the emphysema induced by cigarette smoke in guinea pigs[148]. But none of the previous research has neither evaluated elastin loss with such treatments nor aiming to target elastin specific therapy to improve the mechanical function of the lung.

**Elastase inhibitors:** Another class of drugs that are being used in research are elastase inhibitors. Inhibiting human neutrophil elastase can be a direct treatment to prevent further deterioration of mechanical function of lungs. Many earlier studies have been documented with various elastase inhibitors in the past [149-152]. A study with succinyl-alanyl-alanyl-prolyl-valine-chloromethyl ketone (CMK) in hamsters showed CMK protected against emphysema when instilled 1 hour before human neutrophil elastase instillation [153]. Pre-treatment of hamsters with FR901277, an elastase inhibitor has been reported to reduce lung functional parameters quasi-static compliance and vital capacity in a hamster model of elastase induced emphysema [154]. ZD0892, a synthetic serine elastase inhibitor, showed reduction in inflammation levels and desmosine levels were also returned to control values showing increased elastin content [155]. So far, Sivelestat sodium hydrate (ONO-5046) is the only HNE inhibitor that is in use, but its use is highly limited owing to
its organ toxicity and irreversible inhibition of human neutrophil elastase (HNE) [156-159]. In this perspective, a potential neutrophil elastase inhibitor can definitely have a key role in preserving elastin in lungs.

Alpha-1 anti-trypsin deficiency (AATD) is a minor cause of emphysema. Its main role is maintaining a balance between protease and anti-protease activity by inhibiting the proteolytic enzymes like neutrophil elastase [160, 161]. In the absence of AAT, elastase activity increases and causes elastin breakdown in lungs. For people with AATD, delivery of this protein has been investigated as a possible option to ameliorate the lung physiological function. Purified alpha-1 anti-trypsin (AAT) can come from sources like plasma derived from healthy individuals and recombinant protein [162]. Prolastin is a pioneer product of purified AAT from serum of healthy individuals [163]. Early trials conducted in the late 1990s have all reported that patients who received Prolastin had lower mortality and reduced decline in lung function [164-166]. With the recent advances in gene delivery, it has been made possible to deliver AAT using this method using various viral vectors [167]. Murine model of cigarette smoke induced emphysema has shown improvement with the usage of inhaled recombinant AAT. When treated for 6 months, the animals showed 73% reduction in air space enlargement and also had lower neutrophil levels in the bronchoalveolar lavage fluid (BALF) [168]. Thus, delivery of AAT may be considered as a useful treatment for preserving elastin by inhibiting neutrophil elastase activity in patients with emphysema.
Besides the aforementioned treatments, some novel treatment strategies are being explored. Saluja et al., have reported the efficacy of low molecular weight lignins with respect to anti-elastase, anti-oxidative and anti-inflammatory activities [169]. Shigemura et al., reported amelioration of emphysema by gene transfection of hepatocyte growth factor in Sprague-Dawley rats subjected to elastase instillation [170]. Cruz et al., have documented protective effects of bone marrow derived mononuclear cell therapy in an elastase-induced model of emphysema [171]. In a different treatment approach, Lee et al., have investigated the effects of a herbal formula PM014 in a murine model and have observed that the protective agent used attenuated the increased accumulation of immune cells, TNF-α and IL-6 levels in BALF compared to control mice [172]. Bai et al., reported erythromycin improving the T-cell responses contributing to lower levels of IL-8 and TNF-α in cigarette smoke induced lung inflammation [173].

1.14 Drug delivery to lungs

Drug delivery to lungs has been investigated for centuries. In India, the Ayurvedic practice of making inhalation of stramonium and hemp by pipe was developing but may even have had its origins earlier than the ancient Egyptian use of inhalers. The Ebers papyrus from ancient Egypt (1554 B.C.) describes how the breathless may be treated by the inhalation of the vapor of black henbane [174]. With recent advancements, instruments like metered dose inhalers and nebulizers have conquered the drug delivery through inhalation route to treat pulmonary diseases. With respect to the vehicle for drug delivery, solid lipid nanoparticles, liposomes, and polymers have long been exploited as an option for controlled drug delivery.
Solid lipid nanoparticles

Solid lipid nanoparticles (SLN) are nanosized aqueous suspensions made of primarily triglycerides and phospholipids [175]. Silva et al., have used quercetin loaded SLNs as an anti-oxidant and anti-inflammatory therapy for asthma [176]. In a study by Paranjpe et al., sildenafil loaded SLN was tested for its toxicological effects in heart and lung tissues [177]. Also, curcumin loaded stearic acid and lecithin based SLN was investigated as a treatment for asthma by Wang et al., [178]. Amikacin, an antibiotic, was used with cholesterol SLNs for treatment of lung infections. They observed that SLNs showed better lung deposition and lasted longer when administered using pulmonary route [179].

Liposomes

Liposomes are another lipid-based vehicle used for drug delivery. In the treatment of pulmonary diseases Arikace® (Amikacin,Insmed, Monmouth Junction, NJ, USA) and Pulmaquin™ (ciprofloxacin, Aradigm Corp., Hayward, CA, USA) have advanced to clinical trials aiming at treating lung infections [180]. Liu et al., have demonstrated higher drug targeting efficiency of ciprofloxacin when delivered using phospholipid based liposomal formulation, as compared to ciprofloxacin solution alone [181]. Liposomes for delivery of other drugs like anti-oxidants, anti-inflammatory drugs have also been documented in the literature [182] [183].
**Polymeric nanoparticles**

Owing to the possibility of tailoring of physical, surface, and degradation characteristics, nanoparticles made of polymers like poly (lactic acid) (PLA), poly ethylene glycol (PEG) present themselves as lucrative options in pulmonary drug delivery. In a study by Gill et al., [184] paclitaxel loaded PEG micelles were used for drug delivery. They found better drug absorption and localization in the lungs compared to systemic delivery of commercially available taxol. In a study by Yoo et al., a novel anti-inflammatory compound, hydroxybenzyl alcohol (HBA) was delivered using polyoxalate (HPOX) nanoparticles. They observed attenuation in the inflammatory response by a decrease in the levels of pro-inflammatory cytokines in the ovalbumin induced asthma mice [185]. Nanoparticles also provide a wide array of targeted delivery opportunities [186], which can increase availability of drug and systemic effects of the same. Other drug delivery vehicles like dendrimers, magnetic nanoparticles, colloidal dispersions have also been characterized and used by researchers to deliver drugs [187, 188]. One interesting observation that relates to COPD is work by Sivadas et al., [189]. They developed micro particles based on alginate polymer and elastin. These micro particles respond to elastases which prove to be useful in COPD related applications.

### 1.15 Barriers for drug development for COPD

There are no drugs available to arrest early stage emphysema despite availability of many anti-inflammatory drugs. The attempt to develop a long-term treatment for emphysema encounters two major problems. First, as chronic bronchitis is seen to co-exist
with emphysema in COPD, the primary concern of the treatments available now is to provide a relief to patients with their breathing with bronchodilators [139]. The current therapeutic approach also aims to mitigate the inflammation using corticosteroids, phosphodiesterase-4 inhibitors like Theophylline, MAPK inhibitors (SD-282 ), inhibitors of PI3Kγ/δ and even macrolide antibiotics like Azithromycin[190] [191]. It is important to understand that decreasing the inflammation does not necessarily compensate the lung capacity that has been lost in terms of elastic recoil. At this point in COPD research, the loss associated with the disease is permanent. The loss of elastin in the alveolar walls results in decreased surface area for gas exchange and thus cannot be repaired fully. Secondly, the general route of administration of drug for pulmonary applications is inhalation. Inhalation of either the drug alone or drug loaded carrier particles face a lot of challenges in delivering drug to lungs. Emphysema involves the loss of inter-alveolar septa, which likely decreases the pulmonary uptake of drug due to the available surface area reduction [192]. Obstructed airway physiology, mucus plugs in bronchi and bronchioles may lower the deposition of aerosolized drug in the diseased areas which reduces the therapeutic effect [193]. Even in normally functioning lungs, a drug that has been inhaled may get eliminated by one of the three processes viz. mucociliary or cough clearance to the gastrointestinal tract, by passive or active absorption into the capillary blood network, or by metabolism in the mucus or lung tissue. The fate of an inhaled particle depends on its size, anatomy of airways and ventilatory parameters [194]. Nanoparticle have recently been investigated as potential drug delivery systems to the lungs because of various advantages they offer [195]. In order to increase the specificity and availability of nanoparticles loaded with drugs, use of
monoclonal antibodies to specific biomolecules within the lungs has been suggested as a potential opportunity but has been poorly explored [186, 196]. There is a greater need for a highly effective drug-delivery method, which combines an active targeting formulation and consistent local release of a drug for enhanced therapeutic effect for COPD.

1.16 Polyphenols as enzyme inhibitor, anti-oxidant, and elastin stabilizing agents

Polyphenols are a large and varied family of natural substances. Polyphenols consist of a hydrophobic core surrounded by phenolic (-OH) groups on the exterior of the compound. They can also be divided into ligands, flavonoids, phenolic acids, and stilbenes based on the number of phenolic rings and their accompanying structural elements [197]. They are present in all vascular plants and are secondary metabolites.

Pentagalloyl glucose (PGG), a derivative of tannic acid (TA) is a polyphenol found in green tea and wine [198]. PGG (Figure 1-9) is comprised of five identical ester linkages which involve the core sugar’s aliphatic hydroxyl groups. PGG’s alpha anomer is not common in nature, but PGG does have numerous isomers, as do the rest of gallotannins. All of these isomers have a molecular weight of 940 g/mol. What vary based on the different structures of the isomers are the biochemical properties, specifically the ability to precipitate proteins, susceptibility to hydrolysis, and chromatographic behavior. While PGG is best known for its anti-oxidant properties, its elasto-regenerative properties have been demonstrated both in vivo and in vitro [199-201].
Polyphenols possess many beneficial properties that allow us to consider them as a potential treatment option for COPD. Polyphenols have long been characterized with respect to their protein interactions and protein precipitating ability [202]. Isenburg et al., have found that PGG can stabilize elastin and protect elastin fiber from further damage. It binds to elastin by hydrophobic interactions and makes it resistant to degradation by elastases [200]. Sinha et al., have observed that polyphenols not only protect elastin from degradation but also increases insoluble elastin production in healthy and aneurysmal vascular smooth muscle cells in vitro as shown recently by us [203]. Recent studies on abdominal aortic aneurysm (AAA) model in rats have shown that systemic delivery of PGG loaded NPs can inhibit aneurysm formation and revert aorta back to normal size [204]. Jimenez et al., showed that polyphenols protect elastin from degradation in dermal fibroblast cultures [205]. Evidence of polyphenol affinity towards proline residues concurs on this observation [206]. In addition to protecting elastin, green tea polyphenols have also been shown to inhibit matrix metalloproteinase activity [207, 208]. Other polyphenols like

![Chemical structure of PGG](image)

*Figure 1-9: Chemical structure of PGG*
curcumin and xanthohumol have also been investigated along the lines of elastin stabilization, matrix metalloproteinase activity inhibition and even anti-inflammatory effects [209, 210].
2 PROJECT RATIONALE AND SPECIFIC AIMS

As described in the first chapter, there have been many studies documented to understand the pathophysiological mechanisms of emphysema progression. Many therapies have been tested for stopping the progress of lung damage in animal models. These therapies include usage of inhaled corticosteroids, anti-inflammatories, leukotriene inhibitors, phosphodiesterase inhibitors, synthetic MMP inhibitors etc., in an effort to stop the progression of lung damage. Other studies have used carboxymethyl cysteine, N-acetylcyesteine, polyphenols like curcumin as preventive therapeutics, which successfully showed that prior treatment of lungs with such compounds could restrict lung parenchymal damage. However, none of the therapies target the preservation and regeneration of elastin as a treatment to reverse emphysema. Given the direct and easy route for the drug to reach lungs, researchers have concentrated mostly on inhalation therapeutics. While this remains an easier option, the airway pathology, patients’ ability in inhaling the drug and drug clearance mechanisms in lung play a key role in determining the therapeutic efficiency of the drug. Targeted drug delivery to lungs through systemic route has not been explored to the maximum extent.

As shown earlier, one of the important cause of emphysema’s worsening is loss of elastin in lung alveoli. We believe stabilizing elastin and making it resistant to degradation can halt the progression of emphysema. Furthermore, if we can restore lost elastin by targeted therapy, we can even reverse the disease. We have shown that pentagalloyl glucose (PGG) binds to elastin and inhibits its degradation by proteases [211] in cardiovascular
tissues. It binds to elastin by hydrophobic interactions and makes it resistant to degradation by elastases [200]. We have shown that periadventitial administration of PGG inhibits the development and further progression of already developed AAA in calcium chloride injury rat model [211]. We have further shown in cell cultures that PGG not only protect elastin from degradation but it also increases insoluble elastin production in healthy and aneurysmal vascular smooth muscle cells [203]. Polyphenols including PGG, epigallocatechingallate (EGCG) and Catechin were shown to increase coacervation of tropoelastin and LOX activity and thus increase insoluble cross-linked elastin deposition. Additionally, polyphenol treatments also decreased cellular MMP-2 activity as detected by zymography. Recent studies from our lab on AAA model in rats have shown that systemic delivery of PGG loaded NPs can inhibit aneurysm formation and revert aorta back to normal size [204].

Thus, we would like to first test if inhibition of MMP activity with targeted MMP inhibitor therapy can prevent emphysema development. Next we would like to test if targeted delivery of PGG to emphysematous lungs can prevent progression of emphysema. By stabilizing the elastin in lungs, the connective tissue damage can be controlled along with a possibility of restoring the elasticity. PGG not only stabilizes and regenerates elastin but it also inhibits MMPs. It is known for its anti-oxidant properties also. Thus, PGG can be considered as a multi-purpose drug. As the earliest marker of emphysema is elastin degradation in lungs, developing nanoparticles that can only target degraded elastin would be a superior approach. We further hypothesize that these targeted NPs will stay at the site of damage for longer time and release the drug in a controlled fashion. Such a strategy,
once developed, can be used for other lung disorders to deliver minimal doses of drugs right where they are needed.

2.1 Innovation of current research

Use of bronchodilators and a few anti-inflammatory drugs might help alleviate the difficulties experienced by patients but there have not been any successful long-term therapeutics. The innovation of this research comes from two new approaches. One is to develop a drug delivery strategy and specifically deliver it to damaged lungs thereby allowing an increased drug availability and efficiency. This will greatly reduce drug dosage required which is critical when it comes to MMP inhibitors such as doxycycline. Secondly, the targeted delivery of a naturally occurring polyphenol PGG with nanoparticles will serve a dual-purpose of keeping inflammation and oxidative damage in check and allowing elastin regeneration in lung alveoli.

SPECIFIC AIM 1: To investigate whether elastin-antibody conjugated albumin nanoparticles can be targeted to emphysematous lungs.

Approach: We use intra-tracheal injection of elastase to create emphysema in rats. We then perform initial targeting studies with DiR (1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindotricarbocyanine iodide) dye loaded bovine serum albumin (BSA) nanoparticles (NPs) that are conjugated with anti-elastin antibody. We study the targeting percentages in various organs; analyze the lungs at histological level to study parenchymal damage and elastin damage.
**SPECIFIC AIM 2:** To investigate whether targeted nanoparticles can deliver an active drug like doxycycline to lungs and prevent MMP activity and elastin degradation.

Approach: First we optimize loading of doxycycline in BSA-NPs that are conjugated with anti-elastin antibody. We study drug loading efficiency, *in vitro* release, drug activity, and cellular uptake of NPs. NPs with optimum drug loading and release profile are then delivered intravenously in rats where lungs have been damaged using intra-tracheal elastase injection. We study the MMP activity from the BALF of rat lungs and compare them among control, nanoparticle and systemically treated groups.

**SPECIFIC AIM 3:** To investigate whether PGG treatment can help pulmonary fibroblasts make more elastin while suppressing MMP activity in a cell culture model of emphysema

Approach: We create emphysema-like inflammatory conditions by treating rat pulmonary fibroblasts in cell cultures with TNF-α and CSE. Elastin quantification, MMP activity in the medium, LOX activity quantification combined with gene expression studies of elastin and LOX is included in the experimental design to understand if PGG can increase elastin deposition. We then also look at the reactive oxygen species (ROS) in the cells exposed to TNF-α and CSE and anti-oxidant property of PGG treatment.
**SPECIFIC AIM 4:** To investigate whether PGG loaded NPs can be targeted to emphysematous lungs and whether they increase elastin matrix deposition, thus bringing back the elastic recoil of lungs.

*Approach:* Here we create a mild emphysema by elastase challenge in mice. We investigate the progression of disease at different time points after treatment with PGG loaded NPs. We use FinePointe® Resistance and Compliance machine to track the changes in lungs’ mechanical properties, as a measure of disease progression or suppression. Histological analyses then serve as an aid to observe the elastin stabilization/regeneration in lungs. Finally, we also look at local MMP activity quantification to assess MMP inhibition *in vivo* by PGG nanoparticles.

Each specific aim research is described in separate chapters below.
3 SPECIFIC AIM 1: TO INVESTIGATE WHETHER ELASTIN-ANTIBODY CONJUGATED ALBUMIN NANOPARTICLES CAN BE TARGETED TO EMPHYSEMATOUS LUNGS

3.1 Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in U.S. following cancer and heart disease. Chronic smoking has been established as a major risk factor, while recent research unveiled a possible genetic predisposition factor that could be associated with the development of clinically significant disease [13-16]. Emphysema is one of the two chronic pathological conditions seen in COPD patients. Currently, the widely accepted hypothesis about emphysema is that chronic exposure to cigarette smoke and particulate matter causes irritation in the alveoli and this triggers an inflammatory response. This response attracts many types of inflammatory cells like macrophages, neutrophils, and lymphocytes. Cytokines, reactive oxygen species, prostaglandins, leukotrienes, proteases mediate the progression of the disease [212, 213].

The most general route of drug delivery used for pulmonary applications is inhalation of drug. Due to the lack of active targeting drug delivery system, the drugs that are inhaled into the lungs become restricted in terms of their availability over time. Particles with a size of 5-10 microns get deposited in mouth and larger airways by impaction mechanism. Those with size of 1-5 microns are deposited in smaller bronchi and 1-3 micron sized particles can go deeper up to bronchioles via sedimentation. Particles with size 0.5-1 micron get deposited in the alveoli [214-216]. Apart from size, factors like hydrophilicity/hydrophobicity of the drug, mucociliary clearance mechanisms, drug
metabolism in lungs dictate the fate of inhaled drug. Natural clearance mechanisms like coughing and phagocytosis can prevent the drug from reaching the alveoli. From emphysema point of view, where the elastin damage is present in the innermost structures of the lung, there is a need for more drug to be released locally to stabilize and regenerate the lost elastin.

In this research aim, we have used the elastin degradation in emphysema to target our DiR drug loaded bovine serum albumin (BSA) nanoparticles (NPs) using anti-elastin antibody coating. This antibody coated nanoparticles target only damaged elastin, while sparing the healthy elastin in the body and stick to the targeted site for a longer period.

3.2 Materials and Methods

3.2.1 Preparation of DiR dye loaded BSA nanoparticles

DiR dye (PromoCell GmbH, Heidelberg, Germany) loaded BSA (Seracare, Milford, MA) nanoparticles were prepared using desolvation method and conjugated to anti-elastin antibody (US Biological, MA, USA) for targeting purposes described previously [217-220]. Briefly, 250 mg of BSA was dissolved in 4ml of DI water. 2.5 mg of DiR dye dissolved in acetone was added to BSA solution. The mixture was stirred for one hour at room temperature following the addition of glutaraldehyde (EM grade 70%, EMS, PA, USA) at a concentration of 42µg/mg BSA. The mixture was added dropwise to 24ml of ethanol while sonicating (Omni Ruptor 400 Ultrasonic Homogenizer, Omni International Inc, Kennesaw, GA). The mixture was sonicated on ice for 30 minutes. Thus obtained DiR-
BSA NPs were separated by centrifugation at 10,000 rpm for 10 minutes and washed with water by resuspension.

### 3.2.2 Tagging NPs with elastin antibody

DiR-BSA NPs were PEGylated (mPEG-NHS, PEG succinimidyl ester, MW 2000) (Nanocs, New York, NY) by incubating 2.5mg of PEG with 10mg of nanoparticles at room temperature for one hour with gentle vortexing. The elastin antibody (United States Biological, Swampscott, MA) was thiolated using Traut’s reagent. 1 mg/ml of Traut’s...
reagent prepared in HEPES buffer at pH 8.8. 10µg of elastin antibody was added to 34 µl of 1mg/ml Traut’s reagent and 400µl of HEPES buffer. The thiolation process was carried out at room temperature for 1 hour, followed by filtration through 30kDa MWCO filter at a centrifugation speed of 10000g for 1 min. Finally, the thiolated antibody and PEGylated NPs were combined and incubated on a rocker shaker overnight at 4°C, to obtain ELN-DiR-BSA NPs. Control particles were tagged with the IgG (Sigma Aldrich, St. Louis, MO) antibody (IgG-DiR-BSA NPs). A schematic diagram of conjugating DiR-BSA NPs to elastin antibody is shown in Figure 3-1.

3.2.3 In vitro nanoparticle uptake

ELN-DiR-BSA NPs were tested for their uptake by cells in a cell culture setting. Rat alveolar macrophages, rat pulmonary fibroblasts and human vascular endothelial cells (HuVECs) were grown in 24-well cell culture plates and subsequently incubated with ELN-DiR-BSA NPs (1 mg/ml) at 37°C in 5% CO2 for 24 hours. Two different batches of NPs were prepared as controls for charge and particle size. One batch was prepared with a positive surface charge (POS-DiR-BSA NPs), and one batch was prepared with smaller particle size (SMA-DiR-BSA NPs). Positive surface charge was created by coating the NPs with chitosan deacetylated [221], average low molecular weight (Sigma, St. Louis, MO). 6 µg glutaraldehyde per mg BSA was used to produce NPs with a smaller particle size. Cells were washed afterwards with sterile phosphate buffered saline (PBS) for a total of three washes. Cells were imaged using EVOS® XL Cell Imaging System to visualize NP uptake.
3.2.4 Targeted delivery of ELN-DiR-BSA NPs *in vivo*

All animal studies were performed according to the approved protocols and were compliant with the rules and regulations of Clemson University’s Institutional Animal Care and Usage Committee (IACUC) at all times. To induce elastin damage and emphysema, one of the widely established models i.e., elastase model of emphysema was chosen. Six week old male Sprague- Dawley (SD) rats (n=7) were used for *in vivo* targeting study. Four of the rats were given 50U of porcine pancreatic elastase (PPE) (Elastin Products Company Inc., Owensville, MO) dissolved in 200ul of phosphate buffered saline (PBS) and filter sterilized via intra-tracheal instillation. Remaining three rats received same volume of PBS. The rats were then allowed to undergo damage over four weeks of time after which they were injected with DiR loaded nanoparticles via tail vein. Three elastase and saline rats received ELN-DiR-BSA NPs. One elastase injected rat received IgG-DiR-BSA NPs to eliminate the non-specific targeting variable. Twenty-four hours after injection of nanoparticles, rats were euthanized (Figure 3-2).

![Figure 3-2: Timeline graph of study involving targeted delivery of DiR-BSA NPs in rats.](image)
3.2.5 Lung compliance measurement

Lung compliance was measured manually, post-mortem. Briefly, the trachea was cannulated and tied off with suture. A known volume of air was delivered to inflate the lung and the pressure was recorded using a pneumotach.

3.2.6 DiR signal measurement

After lung compliance measurement lungs, liver, aorta, heart, kidneys and spleen were harvested to check for DiR signal. This was done by imaging each organ with IVIS® Lumina XR Imaging system (Caliper Life Sciences, Waltham, MA) set to excitation/emission of 745nm/790nm. Background signal was subtracted before analyzing the signal intensities from the organs. Bio-distribution of nanoparticles was calculated based on the equation below,

\[
\text{Percentage Biodistribution} = \left( \frac{\text{Fluorescence in organ of interest}}{\text{Total fluorescence in all organs harvested}} \right) \times \frac{\text{Dry weight of organ of interest}}{\text{Total dry weight of all organs}} \times 100\% 
\]

Then the lungs were perfusion fixed using neutral buffered formalin and stored for histological analysis.

3.2.7 Histological analysis

Sections from both right and left lungs were used in observing damage in lungs. Processed tissue samples were embedded in paraffin and sections of 5 μm thick were made from the sagittal face. Hemotoxylin and Eosin (H&E) staining was used to observe general structural characteristics and enlargement of alveoli. Verhoff’s Van Gieson (VVG) staining (Polysciences Inc., Warrington, PA) was performed according to manufacturer’s protocol to
look at elastin damage in the alveolar walls of the tissue. Furthermore, perfusion fixed lungs were cut into small pieces and embedded in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (ThermoScientific, Waltham, MA). 5 μm sections were made with a cryostat and observed using a CY-7 filter cube with EVOS® XL Cell Imaging System.

### 3.2.8 Statistical analysis

All *in vitro* experiments were done in triplicates. For *in vivo* studies, three to four animals were used per group. Data were analyzed by one-way ANOVA followed by Tukey’s test or Dunnett’s test. Dunnett’s procedure was used when comparing all other treatments to a reference and Tukey’s was used for all pairwise comparison. The data are reported as the mean ± standard deviation. Results were considered to be significant with \( p \leq 0.05 \).
3.3 Results

3.3.1 Nanoparticle uptake

Nanoparticles were investigated for their uptake by cells in vitro. This included incubating cells with three different types of cells. Normal DiR-BSA NPs were not taken up by any of the three cell types since they possess negative charge on their surface. But positively charged (POS-DiR-BSA NPs) and small size NPs (SMA-DiR-BSA NPs) were

![Figure 3-3: Nanoparticle uptake of DiR-BSA NPs. Normal NPs were not internalized by cells while POS-DiR-BSA NPs and SMA-DiR-BSA NPs were readily taken up. Purple-DiR nanoparticles imaged using Cy7 filter. Scale bars: A-G 400µm; H-I 100 µm.](image)
readily taken up by all the cells (Figure 3-3). Alveolar macrophages seemed to exhibit more uptake compared to fibroblasts and endothelial cells.

3.3.2 *In vivo* targeting study

DiR-BSA NPs were tested for their specific damaged elastin targeting *in vivo*. Lung compliance between groups did not show a significant difference (data not shown) which indicated that there was no severe damage done to the lungs. This concurs with the observation that the elastase instilled rats did not experience labored breathing at the end of 4 weeks. Rats which received PBS showed no fluorescence signal in their lungs. On the other hand, elastase instilled rats’ lungs were full of DiR signal (Figure 3-4A). Aorta which acted as a positive control for elastin did not have any signal from the dye in both groups (Figure 3-4B).

![Figure 3-4: NP targeting to elastase damaged lungs. (A) Fluorescence images of saline and elastase instilled rat lungs. ELN-DiR-BSA NPs targeting to damaged elastin in the lungs was clearly seen in elastase group but absent in saline group. (B) Fluorescence images of saline and elastase instilled rat aortae. Aortae from both groups showed no signal confirming the targeting of nanoparticles.](image-url)
Furthermore, the rat that received IgG coating to DiR-BSA NPs did not show any targeting in the lungs. Targeting percentage for organs harvested was calculated by normalizing the epifluorescence signal to average dry weight of the organ. Compared to control group elastase group lungs had about six and half times the signal captured (0.907±0.47% vs 6.06±2.14%) (Figure 3-5A). Liver, spleen and kidneys showed DiR signal in both the groups. Biodistribution of nanoparticles in these organs is shown in Figure 3-5B.

![Biodistribution of NPs](image)

**Figure 3-5**: Bar chart showing biodistribution of NPs (A) Bar chart showing nanoparticle targeting counts in saline and elastase treated rat lungs. (B) Bar chart showing distribution of nanoparticles in liver, kidneys and spleen in both groups. * represents significantly different from saline group.

### 3.3.3 Histology

The damage in lungs was confirmed using H&E staining of histological sections. H&E stain showed areas of air space enlargement in the elastase group rats (Figure 3-6 A-B). VVG staining for elastin revealed damaged elastin in the alveolar walls which could be seen as faded black fibers (Figure 3-6 C-D). Rat lung sections from elastase group showed
ELN-DiR-BSA NPs presence in the alveoli when viewed using CY-7 filter. This also confirmed the targeting of nanoparticles to lungs (Figure 3-7).

Figure 3-6: Histological examination of saline and elastase group rat lungs. (A) saline group rat lung showed no damage while elastase treated (B) rat lung showed air space enlargement (scale bar = 100 µm); (C) saline group rat lung showing no elastin damage while (D) elastase group rat lung showed loss of elastin along the alveolar walls (scale bar=20 µm).
Emphysema is responsible for damage of alveoli owing to the chronic inflammation in the lungs thereby decreasing gas exchange capability of lungs. It also leads to the loss of elastin fibers that results in loss of elastic recoil of lungs making the alveoli collapse. This requires the patient to use greater force to exhale the air out. As for the available treatments are considered, they aim at providing relief to the patients by bronchodilation while new anti-inflammatory agents are being developed [222]. The most general route of administration of drugs for pulmonary applications is by inhalation. There are many advantages of this route like local delivery, availability of drug, needleless treatment etc. but at the same time it may not be suitable for people with diseases like emphysema owing to multiple factors. It has been shown that inspirational capacity of COPD patients is less
than that of normal subjects which might have a role on drug deposition [223, 224]. Inhaled drug particles also have to survive phagocytosis by 12-14 alveolar macrophages which span each alveolus [225]. In silico models are increasingly being used to assess particle deposition profiles. One recent study has shown that, while modeling lobar distribution of particles, differences do exist between normal and emphysematous mice with factors like airway collapse, smaller airways, particle transport through expiration may impact particle deposition [226]. There have been specifically tailored improvements to increase the bioavailability of the drug by overcoming each of these barriers but there still needs a lot of work to be done in attaining a reliable solution to these barriers [227].

Thus, targeting lungs via a systemic route like intravenous administration of nanoparticles seems promising. Elastin damage is one of the characteristics observed at protein level in emphysema. Matured elastic fibers have amorphous elastin protein wrapped around by microfibrils and other proteins which stabilize them. Damaged elastin fibers lack these microfibrils covering them and have the amorphous elastin exposed to elastin degrading enzymes [228-232]. This distinction observed in elastin related diseases has been taken advantage of in preparing nanoparticles to target tissues that experience elastin loss as a part of their pathophysiology. Anti-elastin coated nanoparticles have shown successful targeting in experiments conducted before in our lab with abdominal aortic aneurysm induced rats [218, 220, 233]. Extending this targeting concept to emphysema is investigated in this paper. Matrix metalloproteinases, especially MMP 9 and MMP 12, have been shown to play a central role in extracellular matrix degradation and progression of
emphysema [234, 235] and their inhibition has shown protection against emphysema development in animal models [146, 236].

We did not observe any significant changes in the lung compliance between the groups. There was also no difference observed between the mean linear intercept between the groups. This might be because of the dosage that has been administered to the rats (50U per animal). This is comparatively a low dosage administered to damage the lungs. Massaro et al., [237], while observing alveogenesis using retinoic acid also have used a relatively higher dosage of 2U/g body weight of male SD rats. From the works of Borzone et al., [238] and Corteling et al., [239] it could be noticed that rats are relatively resistant towards developing emphysema compared to mice and hamsters. Rubio et al., [240] have used 75IU of PPE in Wistar rats to show the therapeutic effect of N-acetylcysteine in attenuating the lesions. This shows that selection of species and strain can be important in correlating the extent of damage that would be observed in the lungs.

Our nanoparticles possessed required surface charge and size to not being phagocytosed by macrophages. They were also PEGylated to increase their circulation time in the body. Nanoparticle uptake study showed that due to the negative surface charge these nanoparticles avoid being phagocytosed by macrophages and also uptake by any other cells like fibroblasts and endothelial cells. This result was important in the sense that phagocytosis by alveolar macrophages is one of the clearance mechanisms adopted by the lungs to get rid of foreign particles. Normal DiR-BSA NPs escaped phagocytosis while positive charge and small size DiR-BSA NPs could be clearly seen as internalized by
alveolar macrophages. Cytotoxicity of doxycycline nanoparticles assessed using live dead assay showed no decrease in cell viability. As for the targeting, the significant DiR signal observed from elastase damaged lungs combined with the observation of aortae in the same rats being void of signal proved that the nanoparticles target only damaged elastin in lungs. Adding to this, cryosections of lung sections also showed DiR loaded nanoparticles attached to damaged parts of the alveoli. These targeting studies are in agreement with targeting of aorta in our previous works and could only further reinforce the specificity of these nanoparticles for damaged elastin.

3.5 Conclusion

In conclusion, by taking advantage of elastin damage that occurs in emphysema as a part of its pathological process, we have developed a novel bio-degradable polymeric nanoparticle system that targets specifically elastin damage in lungs. We have confirmed the targeting specificity of these particles using fluorescence imaging of organs and histological examination to locate nanoparticles.
4 SPECIFIC AIM 2: TO INVESTIGATE WHETHER TARGETED NANOPARTICLES CAN DELIVER ACTIVE DOXYCYCLINE TO LUNGS AND INHIBIT MMP ACTIVITY

4.1 Introduction

Emphysema results in poorly reversible airway obstruction due to the destruction of alveoli and elastin fibers in the lung [241, 242]. Loss of elastin in the lungs has been shown to be correlated to loss of lung function in patients [60, 243]. Currently available treatments for COPD aim at only providing temporary relief to the patients by mitigating inflammation or by the action of bronchodilators [139, 141, 142, 191]. Bronchodilators are the best available option which can relax the airway muscles and provide relief from exacerbations of the disease. Corticosteroids have been shown to have no anti-inflammatory effect in COPD patients [190]. New treatments that have come into light include Phosphodiesterase 4 inhibitors like Theophylline [244, 245], Cilomilast, Roflumilast, Tetomilast [246-248], MAPK inhibitors (SD-282 [249]), inhibitors of PI3Kγ/δ [250] and even macrolide antibiotics like Azithromycin [251, 252]. Despite the plethora of drug delivery opportunities available, there is a greater need for a highly effective delivery method which combines an active targeting formulation and consistent local release of a drug for enhanced therapeutic effect.

Matrix metalloproteinases (MMPs) have acquired a lot of attention in emphysema research with their key role of damaging elastin fibers, thus contributing to the enlargement of air spaces and loss of elastic recoil in the lungs [253]. Various MMP inhibitors can also be included in the new set of treatments that researchers are targeting to reduce
inflammation in the lungs [146, 148]. MMP inhibitors have proven to be useful in diseases like abdominal aortic aneurysm and cancer successfully [218, 254-256]. Use of MMP inhibitors in COPD has also been explored in animal models [147, 148]. Doxycycline is one such potential broad spectrum MMP inhibitor that is shown to reduce MMP activity in smooth muscle cells [257, 258]. But there is always the risk of over-targeting MMPs with the systemic delivery of these drugs [259]. So there needs to be a controlled and targeted release of drugs to achieve maximum therapeutic efficiency.

In this aim, we investigated a novel method of drug delivery with a systemic injection of nanoparticles loaded with doxycycline. We took advantage of elastin damage in emphysema and tested our hypothesis that anti-elastin coated bovine serum albumin nanoparticles injected intravenously will target the lungs and with a consistent release of doxycycline over a period, will suppress MMP activity locally.

4.2 Materials and Methods

4.2.1 Preparation of Doxycycline loaded BSA nanoparticles (DOX-BSA NPs)

Doxycycline hyclate (Sigma Aldrich, St. Louis, MO) loaded BSA nanoparticles were prepared using the procedure described here with modifications. Briefly, 25mg of doxycycline hyclate was dissolved along with 100mg of BSA in 2ml of water and allowed to stir at 500 rpm for 30mins. Following this 4ml of ethanol was added dropwise at a rate of 1ml/min using an automated dispenser, which makes the solution turbid. 50μl of 8% gluteraldehyde (40ug/mg BSA) was added to crosslink the albumin and the mixture was set to stir for 2 hours at room temperature. Resulting solution was spun at 14000 rpm for
10mins to separate thus formed nanoparticles. Nanoparticles were washed thrice with DI water before proceeding with anti-elastin conjugation. Table 4-1 shows different parameters optimized in obtaining the final working formulation of doxycycline nanoparticles.

Table 4-1. Characterization of doxycycline loaded bovine serum albumin nanoparticles.

<table>
<thead>
<tr>
<th>Drug to Polymer ratio (Drug/Carrier)</th>
<th>Lot #1</th>
<th>Lot #2</th>
<th>Lot #3</th>
<th>Lot #4</th>
<th>Lot #5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Doxycycline Monohydrate)</td>
<td>1:10</td>
<td>1:5</td>
<td>1:4</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>Gluteraldehyde (ug/ing BSA)</td>
<td>42</td>
<td>42</td>
<td>46.8</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Stirring time</td>
<td>60</td>
<td>30</td>
<td>60</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Desolvant to solvent ratio</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sonication time</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>30, 60, 90</td>
<td>120 (stirring instead of sonication)</td>
</tr>
<tr>
<td>Result/comments</td>
<td>Negligible loading of drug. No nanoparticles formed.</td>
<td>No nanoparticles formed.</td>
<td>Loading was 6.77±1.7% Particle size was in the range of 608nm.</td>
<td>Particle sizes were in the range of 458nm, 469nm and 390nm for respective sonication times.</td>
<td>Particle size was in the range of 170-200nm. Zeta potential was -60μV. Loading was 16.77±1.99%</td>
</tr>
</tbody>
</table>

4.2.2 Tagging NPs with elastin antibody

DOX-BSA NPs were conjugated with anti-elastin antibody using the procedure described in chapter 3. DOX-BSA NPs were PEGylated (mPEG-NHS, PEG succinimidyl ester, MW 2000) (Nanocs, New York, NY) by incubating 2.5mg of PEG with 10mg of
nanoparticles at room temperature for one hour. The elastin antibody (United States Biological, Swampscott, MA) was thiolated using Traut’s reagent. 1 mg/ml of Traut’s reagent prepared in HEPES buffer at pH 8.8. 10µg of elastin antibody was added to 34 µl of 1mg/ml Traut’s reagent and 400µl of HEPES buffer. The thiolation process was carried out at room temperature for 1 hour, followed by filtration through 30kDa MWCO filter at a centrifugation speed of 10000g for 1 min. Finally, the thiolated antibody and PEGylated NPs were combined and incubated on a rocker shaker overnight at 4°C, to obtain ELN-DOX-BSA NPs.

4.2.3 Characterization of doxycycline NPs

Size and zeta potential: The size and zeta potential of DOX-BSA NPs was measured using 90Plus Particle Size Analyzer (Brookhaven Instruments Co, Holtsville, NY) with three runs performed per one sample batch. 1mg/ml solution of nanoparticles was used for measuring particle size. The size was also confirmed by scanning electron microscopy of nanoparticles.

Encapsulation efficiency: The supernatant obtained from the washout was used to estimate the amount of free doxycycline. Absorbance at 273nm was measured using a UV spectrophotometer (BioTek Instruments Inc., Winooski, VT) and compared to a previously prepared standard curve. Percentage of total drug added that was encapsulated was calculated using the following equation,
Encapsulation %

\[= \frac{(\text{Amount of drug added} - \text{Amount of drug in washout}) \times 100}{\text{Amount of total drug added}}\]

**Yield:** 5ml of 1N NaOH was added to freshly prepared nanoparticles and stirred for 36hrs to completely dissolve the bovine serum albumin. Then the absorbance of albumin was measured at 280nm (A280) to calculate the amount of BSA present in the nanoparticles. Nanoparticle yield was calculated as total weight of BSA and doxycycline present in nanoparticles over initial amounts added.

Yield percentage

\[= \frac{(\text{Amount of BSA in NPs} + \text{Amount of doxycycline in NPs}) \times 100}{\text{Total amount of BSA and doxycycline added initially}}\]

**Loading:** Percentage of loading of doxycycline in BSA nanoparticles is calculated using the following equation,

\[\text{Loading} \% = \frac{(\text{Amount of doxycycline in NPs}) \times 100}{(\text{Yield} \times \text{Total amount of BSA and doxycycline added initially})}\]

**In vitro release profile:** Freshly prepared DOX-BSA NPs were re-suspended in 1ml of DI water and incubated at 37°C. At specific time points, the nanoparticles were spun and the supernatant was read at 273nm to measure the amount of doxycycline released.
4.2.4 Cell culture for cytotoxicity

Rat primary pulmonary fibroblasts and alveolar macrophages were obtained from CellBiologics (Chicago, IL) and ATCC (Manassas, VA) respectively. Fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) (ScienCell, Carlsbad, CA) supplemented by 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO), 1% Pencillin-Streptomycin (Corning, Corning, NY) and 1% fibroblast growth substrate (ScienCell, Carlsbad, CA). Alveolar macrophages were grown in K-12 medium (ATCC, Manassas, VA) supplemented with 10% FBS and 1% Pencillin-Streptomycin. HuVECs (Life Technologies, Carlsbad, CA) were grown in endothelial cell medium (ECM) (ScienCell, Carlsbad, CA) supplemented with 10% FBS and 1% Penicillin-Streptomycin. Cells with passage numbers two to five are used for in vitro studies.

4.2.5 In vitro cytotoxicity of Doxycycline NPs

A standard live/dead assay was performed to measure the cytotoxicity of NPs. Rat pulmonary fibroblasts, rat alveolar macrophages and human vascular endothelial cells (HuVECs) were cultured with NPs for 24 hours (0.5mg/ml and 1mg/ml). A Live/Dead assay (PromoCell GmbH, Heidelberg, Germany) was performed according to the manufacturer’s protocol to confirm cell viability.

4.2.6 Functional drug release from nanoparticles

To confirm that the drug released from DOX-BSA NPs is functional in inhibiting MMP activity, a simple MMP inhibition assay was performed ex vivo. BALF from rat lungs was used as sample to quantify MMP activity. Uninhibited sample was used as positive
control while one set of samples were incubated with 50 ng of doxycycline released from DOX-BSA NPs. MMP activity was quantified using internally quenched peptide substrates for MMPs 2&9 (Ex/Em = 280/360 nm, MMP Substrate III, Anaspec, CA) and MMP 12 (Ex/Em = 325/393 nm, 390 MMP FRET Substrate V, Anaspec, CA). One milligram of the substrate was dissolved in 50 µl of DMSO, and the solution was diluted in 10 ml of development buffer (50 mM Tris Base, 5 mM CaCl₂•2H₂O, 200 mM NaCl, 0.02% brij 35). 2 µl of the substrate stock solution and 2 µl of the extracted protein were mixed with 96 µl of the development buffer and incubated for one hour at 37°C. A fluorescent plate reader (BioTek Synergy, BioTek, Winooski, VT) was used to read endpoint florescence intensity.

4.2.7 Targeted delivery of ELN-DOX-BSA NPs in vivo

Six week old male Sprague-Dawley rats (n=10) were used for investigating the potential of doxycycline loaded nanoparticles in suppressing the MMP activity when administered systemically. As shown in timeline graph (Figure 4-1), all the rats received intra-tracheal instillation of PPE (50 U per animal). Three groups of animals were used to test the working of DOX-BSA NPs in comparison to the systemic injection of drug as an acute treatment option. Three rats did not receive any kind of treatment after elastase injection (Non-treated group); three rats received systemic injection of doxycycline injection (Non-treated group); three rats received systemic injection of doxycycline injection (Non-treated group); three rats received systemic injection of doxycycline

![Timeline graph for animal study for targeted delivery of DOX-BSA NPs in rats.](image_url)
hyclate solution via tail vein (300 µg per rat dissolved in 200 µl DI water) three days after the elastase administration and continued to receive weekly injections from then onwards (Weekly IV group). Four rats received one-time systemic injection of ELN-DOX-BSA NPs (10 mg/kg of animal) via tail vein only once i.e., three days after elastase administration (DOX-BSA-NP group). All the rats were euthanized after four weeks of treatment. After euthanasia, lungs of rats were cannulated and flushed with saline to obtain bronchoalveolar lavage fluid (BALF).

4.2.8 Measurement of MMP activity in BALF

MMP activity in BALF was measured using internally quenched peptide substrates for MMPs 2&9 (Ex/Em= 280/360 nm, MMP Substrate III, Anaspec, CA) and MMP 12 (Ex/Em = 325/393 nm, 390 MMP FRET Substrate V, Anaspec, CA). One milligram of the substrate was dissolved in 50µl of DMSO, and the solution was diluted in 10ml of development buffer (50 mM Tris Base, 5 mM CaCl\textsubscript{2}•2H\textsubscript{2}O, 200 mM NaCl, 0.02% brij 35). 2µl of the substrate stock solution and 2 µl of the extracted protein were mixed with 96µl of the development buffer and incubated for one hour at 37°C. A fluorescent plate reader was used to read endpoint florescence intensity.

4.2.9 Immunofluorescence for elastin and MMP-9

Immunofluorescence study was done on paraffin embedded rat lung sections to visualize MMP 9 present in the tissue, using standard protocol. Briefly, tissue sections were deparaffinized and heated in 1X citrate buffer (pH 6.0) (Millipore, Billerica, MA) for 30 minutes for antigen retrieval. They were then cooled to room temperature and washed with
wash buffer thrice. 5% BSA solution was used to block the tissue to reduce non-specific binding. Sections were washed twice with PBS and thrice with wash buffer (Enzo Life Sciences Inc., Farmingdale, NY) to remove excess BSA. Then anti-MMP 9 tagged with CY-7 (Bioss Inc., Woburn, MA) was added to the sections at a concentration of 10 µg/ml and incubated overnight at 4°C on a shaker. After washing the sections were cover slipped with Vectashield (Vector Laboratories, Burlingame, CA) on them to preserve the fluorescence.

4.2.10 In situ zymography

To examine activity of MMPs in the lung tissue samples in situ zymography on histological sections was performed. Fluorescently labeled gelatin was used as substrate. Gelatinolytic activity was observed in Non-treated, weekly IV and DOX-BSA-NP group frozen lung sections (6 µm thick) using DQ-gelatin as a substrate (Life Technologies, IL). Cryostat sections of lungs were air-dried for 10 minutes. One part of DQ-gelatin (1 mg/ml of DI water) was mixed with nine part of 1% agarose (Promega, WI.) in PBS containing DAPI (1 µg/ml) (Life Technologies, IL). A drop of the mixture was added to each section and incubated in development buffer (50 mM Tris Base, 5 mM CaCl₂·2H₂O, 200 mM NaCl, 0.02% brij 35) overnight at 37°C. Images were captured using EVOS® XL cell imaging system.

4.2.11 Reverse zymography

Tissue inhibitors of matrix metalloproteinases (TIMPs) activity was analyzed in the tissue homogenate using reverse zymography. Resolving (15% polyacrylamide, 1mg/ml
gelatin supplemented with 20U of collagenase) and stacking (4% polyacrylamide with 1% sodium dodecyl sulphate (SDS)) gel solutions were prepared fresh for every assay. 10% Ammonium persulfate and TEMED were added to polymerize the gels and they were casted using Surecast® (Thermo Fisher, Waltham, MA) gel casting equipment. Equal protein for all samples was loaded per well along with pre-stained molecular weight standards (Precision Plus Protein Standard, Bio-Rad, Hercules, CA). After electrophoresis gels were washed in 2.5% Triton-PBS solution and then developed in buffer (50 mM Tris buffer, pH 7.5 containing 5 mM CaCl$_2$, 200 mM NaCl, 0.02% brij-35) overnight at 37°C. Then gels were stained with Coommasie blue and destained to visualize the active MMP bands using UVP Geldoc It® (UVP, Upland, CA) imager. The bands were analyzed using GelQuant® software.

4.2.12 Casein zymography

Matrix metalloproteinase 12 (MMP-12) activity was analyzed in the tissue homogenate using casein zymography. Resolving (12% polyacrylamide, 1mg/ml casein supplemented) and stacking (4% polyacrylamide with 1% sodium dodecyl sulphate (SDS)) gel solutions were prepared and stored at 4°C. 10% Ammonium persulfate and TEMED were added to polymerize the gels and they were casted using Surecast® (Thermo Fisher, Waltham, MA) gel casting equipment. Since casein protein also forms bands around the same size (25kDa) as that of MMP-12, the casted gel was pre-run once at 90V for 1 hour to remove excess casein. Then equal protein for all samples was loaded per well along with pre-stained molecular weight standards (Precision Plus Protein Standard, Bio-Rad, Hercules, CA). Electrophoresis was carried out at 90V for 80-90 minutes. After
electrophoresis gels were washed in 2.5% Triton-PBS solution and then developed in buffer (50 mM Tris buffer, pH 7.5 containing 5 mM CaCl₂, 200 mM NaCl, 0.02% brij-35) overnight at 37°C. Then gels were stained with Coommasie blue and destained to visualize the active MMP bands using UVP Geldoc It® (UVP, Upland, CA) imager. The bands were analyzed using GelQuant® software.

4.3 Results

4.3.1 DOX-BSA NPs characterization

DOX-BSA NPs were characterized based on their size, zeta potential, yield, loading and release of drug (Table 4-2). DOX-BSA NPs had an average size of 175±39.66 nm. The average size of nanoparticles was also confirmed by SEM images taken (Figure 4-2). Zeta potential of these particles was measured to be -59.8±4.67 mV. Amount of doxycycline in NPs was 7.1±0.709 mg corresponding to an encapsulation efficiency of 32.74±6.3%. Amount of BSA converted into NPs was calculated by dissolving them in 1N NaOH. Absorbance values at 280nm measured BSA in NPs as 31.7±2.09 mg. Total nanoparticle yield was calculated by summing the amounts of doxycycline and BSA present in NPs, leading to a value of 34.71±3.54%. Loading percentage of doxycycline in NPs was calculated using the formula mentioned above and this was equal to 16.77±1.99%.
In vitro release of DOX-BSA NPs doxycycline from the nanoparticles was measured at different time points (Figure 4-3). In the first 24 and 48 hours 9.68±3.4% 13±4.56% of the drug was released. Using the NPs injection dose per animal, loading percentage of doxycycline and targeting percentage of NPs to the lungs in the formula mentioned in methods section, the amount of drug that would be delivered at each time point to the lungs was calculated to be 2.85±1.31 μg in 48 hours while the total drug released from NPs into the system was 1.24±0.57mg. Percentage of doxycycline released from BSA NPs steadily increased and reached 46±9.54% in four weeks. By this time the total amount of drug delivered to the lungs was 9.15±1.38 μg.
Table 4-2 Properties of DOX-BSA NPs

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>175±39.66 nm</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>-59.8±4.67 mV</td>
</tr>
<tr>
<td>Yield percentage</td>
<td>34.71±3.54 %</td>
</tr>
<tr>
<td>Loading percentage</td>
<td>16.77±1.99 %</td>
</tr>
</tbody>
</table>

Figure 4-3: Release curve of doxycycline from DOX-BSA
4.3.2 Cytotoxicity of DOX-BSA NPs

DOX-BSA NPs were not found to be toxic to all the three types of cells tested for at 0.5mg/ml and 1mg/ml concentrations. Live dead assay of all the three types of cells showed no abnormalities in morphology and no dead cells (Figure 4-4). The cell viability was same as that of control group. On the other hand, ethanol group showed no cells.

**Figure 4-4:** In vitro cytotoxicity and nanoparticle uptake by cells. Cell viability of DOX-BSA NPs as assessed by Live-Dead assay. Cell viability was observed for three different types of cells i.e., rat alveolar macrophages, rat pulmonary fibroblasts and human vascular endothelial cells, under four different conditions, i.e., control, 1mg/ml DOX-BSA NPs, 0.5mg/ml DOX-BSA NPs and ethanol (50% final concentration). Scale bar = 400 µm;
4.3.3 Functional drug release from nanoparticles

MMP activity quantification from BALF of rats showed that doxycycline released from the nanoparticles is functional. MMP 2, 9 and 12 activities were significantly suppressed by the released drug from nanoparticles as compared to the uninhibited sample as shown in Figure 4-5.

Figure 4-5: Bar chart showing MMP activity suppression by doxycycline released from nanoparticles * represents significantly different (p<0.05). Dashed line represents uninhibited sample.

4.3.4 Measurement of MMP activity in BALF

To compare the broad spectrum MMP inhibitory effect of doxycycline when delivered through two mechanisms, systemic and targeted, MMP activity in the BALF was measured using specific FRET substrates for MMPs 2&9

Figure 4-6: MMP activity in the BALF measured using specific FRET substrates for MMPs 2, 9 and 12, * represents significantly different from non-treated group (n=4 for DOX-BSA-NP group; p<0.05)
and MMP-12. Weekly IV doxycycline group showed very high MMP 2,9,12 activities. Dox NP group showed a significant reduction in MMP activities (Figure 4-6).

4.3.5 Immunofluorescence to visualize MMP-9

To confirm the inhibition of MMPs by doxycycline an immunofluorescence imaging was performed on lung sections of all groups, as described above. MMPs were almost completely not seen in the groups treated by nanoparticles and acute systemic treatment with doxycycline (Figure 4-7).

![Immunofluorescence assay for MMP 9](image)

Figure 4-7: Immunofluorescence assay for MMP 9 in (A) Non-treated group, (B) Weekly IV group and (C) DOX-BSA-NP group. Purple corresponds to MMP 9. Scale bar=400 µm.

4.3.6 In situ zymography

In situ zymography is used to detect active MMPs in histological sections. When the intensity of fluorescence was compared between weekly IV drug only injection and DOX-NP groups, we observed a significant suppression of signal from DOX NP group’s lung section compared to the IV drug injection (Figure 4-8). Non-treated group also showed similar MMP activity signal on par with Weekly IV group. This reduced MMP
activity concurred with the FRET assay results showing that one nanoparticle injection can suppress MMP activity for an extended period of time.

4.3.7 Reverse zymography

Reverse zymography of control, non-treated and DOX-BSA-NP group samples showed different TIMP activities. DOX-BSA-NP group showed less TIMP activity while the other two groups showed relatively more intense bands on the gel. This could be thought as a possible feedback mechanism by which reduction of MMP activity in the lungs by the released doxycycline might have caused the TIMPs to become not active. On the other hand, Non-treated group showed more TIMP activity to check MMP activity in the lungs.

Figure 4-8: In situ zymography of frozen lung sections from non-treated, Weekly IV and DOX-BSA-NP group rat lungs. Fluorescence due to MMP 2 & 9 mediated breaking down gelatin can be seen in Weekly IV group (green) but reduced in DOX-BSA-NP group. Nuclei are stained with DAPI (blue). Scale bar = 200 µm.
4.3.8 Casein zymography

Analysis of casein zymography gels showed that MMP-12 activity is significantly higher in the non-treated group while DOX-BSA-NP group has significantly reduced MMP-12 activity. Controlled release of doxycycline from the nanoparticles helps keep the activity of MMP-12 low in the lungs (Figure 4-9). This result is in concordance with FRET assay we used above for quantifying MMP activities.

Figure 4-9: Casein zymography images showing MMP-12 activity in Weekly IV and DOX NP groups. Below is a bar chart of densitometry analysis of MMP-12 activity bands quantified using GelQuant software. * represents significantly different from non-treated group.
4.4 Discussion

Matrix metalloproteinases are proteases that degrade a variety of substrates. Owing to protease-antiprotease imbalance in emphysema, the role of these enzymes in degrading elastin and other ECM proteins has been exemplified using many studies. Inhibition of MMP activity in the lungs can lead to slowing down of lung damage that occurs in emphysema. MMPs, especially MMP 12, play a central role in ECM degradation and progression of emphysema [234, 235] and their inhibition has shown protection against emphysema development in animal models [146, 236]. Churg et al. further stressed the importance of MMP-12 in cigarette smoke model of emphysema by showing reduced levels of this protein produced by alveolar macrophages when treated with α-1 anti-trypsin [260]. Using our novel targeted drug delivery method, we investigated if a functional doxycycline can be delivered to inhibit MMPs in elastase challenged rat lungs. Our aim was to specifically deliver a broad spectrum MMP inhibitor like doxycycline, encapsulated in a bio-degradable nanoparticle system, to curb MMP activity. Doxycycline is a potent inhibitor of MMPs especially MMP 8 and 9 [261-263]. Doxycycline has been previously shown to reduce the severity of abdominal aortic aneurysms (AAAs) in various animal models. Manning et al., [264] have shown reduction of AAA formation in Ang-II murine model of the disease. Their study results also coincide with Uitto et al., [265] who also report decreased MMP 2 activity and also decreased MMP 2 gene expression in epithelial cell cultures. Petrinec et al., [266] have observed decreased local MMP 9 production in the abdominal aorta which led to the preservation of elastic matrix in the rats treated with doxycycline injections daily. We have synthesized DOX-BSA-NPs and characterized them
based on their yield, size, zeta potential, drug release and loading properties. Cytotoxicity of doxycycline nanoparticles assessed using live dead assay showed no decrease in cell viability at two different concentrations. These concentrations were very less compared to that delivered to the animal (10mg/kg animal).

BALF is a key indicator of inflammation that is going on in lungs. Measurement of MMP activity from BALF evaluated the efficacy of nanoparticles in delivering functional doxycycline to the lungs. Non-treated rats without any drug treatment showed high MMP activity in BALF. This level of activity was similar to rats receiving weekly injections of doxycycline, clearly showing drug alone was inefficient in inhibiting lung MMPs. We show that targeting of nanoparticles to damaged elastin in the lungs and a controlled release of doxycycline played an important role in keeping down the MMP activity levels in rats that received single DOX-BSA NPs injection. An obvious advantage of such treatment is requirement of lower dosages.

Dalvi et al., [267] have reported significant improvement in lung functions when moderate to severe COPD patients were treated with doxycycline (100 mg per day). In another recent pilot study Bhattacharya et al., [268] have observed pulmonary function improvements with doxycycline treatment (100 mg per day) compared to standard pharmacotherapy. In animal models, Rossiter et al., [269] have showed VEGF LoxP mice instilled through the trachea with an adeno-associated virus expressing Cre recombinase had reduced mean linear intercept values with continuous doxycycline treatment along with reduction in MMPs. Sochor et al., [270] have demonstrated MMP-9 inhibition and thus
pancreatitis-associated lung injury in rats with doxycycline treatment (30 mg/kg). Doroszko et al., [271] have reported MMP 9 inhibition by doxycycline (2 mg/kg) in mechanical ventilation-induced lung injury in Wistar rats. These experiments lasted for less than one day. On a similar scale, the average amount doxycycline released into the body of each rat in 24 hours is 0.163 mg/kg in our current study. This corresponds up to ~180 and ~12-fold decrease in the amount of drug required for MMP inhibition with targeted delivery compared to Sochor et al., and Doroszko et al., results respectively. Doxycycline is used as an example in this paper to pave the way for many other drugs that can be targeted to lungs by our novel NPs. The reduced dosage using targeted delivery can be particularly useful when toxic drugs have to be delivered for treatment of diseases like lung cancers.

### 4.5 Conclusion

In conclusion, by taking advantage of elastin damage that occurs in emphysema as a part of its pathological process, we have developed a novel bio-degradable polymeric nanoparticle system that targets specifically lungs. We have confirmed the targeting specificity of these particles. Doxycycline loaded BSA NPs were optimized in their size, surface properties, yield, drug loading and release properties. Targeted delivery of doxycycline nanoparticles have led to significant inhibition of MMPs in the lungs. This opens up a promising way of controlling inflammation in emphysema thereby stopping further damage to the lungs.
5 SPECIFIC AIM 3: TO INVESTIGATE WHETHER PGG TREATMENT CAN HELP PULMONARY FIBROBLASTS MAKE MORE ELASTIN WHILE SUPPRESSING MMP ACTIVITY IN A CELL CULTURE MODEL OF EMPHYSEMA

5.1 Introduction

Inability to restore lost elastin from the lungs poses a serious hurdle in halting the progression of emphysema in patients. In COPD patients less elastin is shown to be present in the distal lung parenchyma and small airways [59, 60]. Moreover, elastin degradation results in elastin derived peptides (EDPs), which act as chemoattractants for monocytes and other inflammatory cells [272]. Severe COPD patients have shown increased elastin mRNA production in their lungs but without a functional elastin increase [273]. It has also been shown that fibroblasts, which are major players in elastin production in lungs, switch to a pro-inflammatory phenotype [274]. Elastin regeneration requires coordinated expression of all molecules that help in the process, which does not happen in adults. Supporting this view, LOX and LOXL expression levels have been shown to decrease with age[275] [46].

Participating in the protease-antiprotease imbalance in emphysematous lungs, MMPs have recently occupied a key position in remodeling of the ECM. Many varieties of MMPs have now been discovered, with MMP 12 followed by MMP 9 more pronounced in emphysema as they can degrade elastin and collagen in lung connective tissue [28, 145]. Their importance in emphysema has been documented in many studies [117, 146].
Penta galloyl glucose (PGG) is a derivative of tannic acid (TA) found in green tea, red wine, etc. We have previously shown from in vitro and in vivo experiments that PGG can preserve elastin, inhibit MMP activity and also help restore lost elastin in aneurysmal aorta of rats [203, 204, 276]. We hypothesize that PGG treatment to emphysematous lungs can be a multifaceted solution for treating this challenging disease. Given its anti-oxidant and anti-inflammatory properties combined with its affinity to bind to elastin, it can not only render elastin resistant to degradation, but it can also help in the formation of new elastin [198, 277]. In this aim, we investigated the effects of PGG on rat pulmonary fibroblasts in vitro, in terms of elastin production, elastin deposition in the ECM, inhibition of MMP activity, and its remarkable anti-oxidant effects in two different in vitro conditions mimicking emphysema.

5.2 Materials and methods

5.2.1 Preparation of cigarette smoke extract (CSE)

Cigarette smoke extract was prepared using a custom designed instrument. Smoke from commercially available Marlboro® 100’s cigarettes (with filter) was passed through warm PBS. This column length was about 10 cm in length and the time taken to burn one cigarette was maintained at 5 minutes by adjusting the flow of air. Each cigarette was burnt until there was 2 mm left before reaching the filter. Smoke extract obtained by bubbling smoke from two cigarettes in 10 ml of PBS was considered a 100% concentrated solution.
5.2.2 Pulmonary fibroblast cell culture

Primary rat pulmonary fibroblasts (Cell Biologics Inc©, IL, USA) were grown in Dulbecco’s Modified Eagle Medium (DMEM), (ScienCell™, CA, USA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich®, St. Louis, MO), 1% fibroblast growth substrate (ScienCell™, CA, USA), and 1% Penicillin-Streptomycin. Cells from passages 2-6 only were used for all experiments. For all experiments, cells were allowed to grow for one day in normal growth medium before treatment with other compounds.

5.2.3 FASTIN assay

Total insoluble elastin deposited in the cell layers and soluble tropoelastin in the media were quantified using the Fastin™ Elastin assay kit (Biocolor, UK) according to the manufacturer’s protocol. Matrix elastin was normalized to total lysate protein released by the cells, which is assumed to be directly proportional to the total cell count, while the tropoelastin in medium was normalized to the total protein content in the medium.

5.2.4 PGG concentration characterization

To first optimize the concentration of PGG that should be added to cell cultures, cells were grown in 2, 5 and 10 µg/ml final concentrations (n=3 per group) of PGG dissolved in dimethyl sulfoxide (DMSO). The control group for this experiment had an equal volume of DMSO added to the cells. The volume of DMSO was kept at 0.05% final concentration of the total volume of the cell medium in the well plates. Cell viability was assessed using a Live/Dead assay and Picogreen® dsDNA (Life Technologies, Carlsbad, CA) assay. Elastin deposited in cell cultures was quantified using the FASTIN™ Elastin
assay kit (Biocolor, UK) as per manufacturer’s protocol. Results were normalized to the average of control group.

5.2.5 Cigarette smoke extract characterization

To determine the optimum concentration of CSE that should be added to cell cultures, cells were grown in different final concentrations of CSE viz. 0%, 1%, 5%, 10%, 20% and 50%. A Picogreen® dsDNA assay along with a Live/Dead assay were used to calculate the cell viability under the above stated conditions.

5.2.6 In vitro emphysema model

To mimic emphysematous conditions in vitro, cells were treated with tumor necrosis factor (TNF-α) (Peprotech Inc. ®, Rocky Hill, NJ) and CSE. Cells were divided into six groups depending on the combination of substances they were treated with. The groups were: DMEM (DMEM only), PGG (10 µg/ml), tumor necrosis factor (TNF-α) (50 ng/ml), TNF-α (50 ng/ml) + PGG (10 µg/ml), CSE (5% final concentration), and CSE (5%) + PGG (10ug/ml). The same terminology has been used all throughout the paper for discussing the results obtained. Cells were grown under these conditions for up to 21 days, and the medium was replenished twice every week. The cell cultures were analyzed at days 7, 14 and 21 for total protein, total elastin in matrix and medium, collagen in matrix and medium, and MMP activity in the medium.

5.2.7 mRNA extraction and RT-PCR

Cells grown in the aforementioned conditions were lysed at weeks 1 and 2, and mRNA from the cells was extracted using RNeasy RNA extraction kit (Qiagen, Valencia,
CA) as per the manufacturer’s protocol. The quality of the mRNA extracted was confirmed with the A260/A280 ratio as measured by a BioTek Synergy 2 plate reader (BioTek, Winooski, VT). Primers were designed for obtaining cDNA copies of Lysyl Oxidase (LOX), Lysyl Oxidase 1 (LOXL1), Beta-2 Microglobulin (B2MG) (a housekeeping gene) and Elastin (ELN) genes with the sequences shown in Table 5-1 and obtained from Integrated DNA Technologies® (Coralville, IA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in the following steps: 5 minutes at 25°C, 60 minutes at 42°C, and 10 minutes at 72°C using Oligo dT primers from a RT-PCR kit (Promega, Madison, WI) using a Mastercycler® Gradient PCR machine (Eppendorf, Hauppauge, NY).

Table 5-1. Primer sequences and amplicon sizes for genes of interest

<table>
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<th>Gene</th>
<th>Primer sequence</th>
<th>Primer Length</th>
<th>Amplicon size</th>
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<td>5'-CACTACACAGGTGCTGTTTT-3'</td>
<td>17</td>
<td>137</td>
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<td>FWD</td>
<td>5'-AGGCGGTATGCTTCTTT-3'</td>
<td>17</td>
<td>138</td>
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<tr>
<td>Lysyl Oxidase (LOX)</td>
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<td>17</td>
<td>138</td>
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<tr>
<td>Beta (β2) Microglobulin (B2MG)</td>
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5.2.8 Quantitative PCR

PCR amplification of cDNA was performed in the following sequence: 1 minute at 95°C, 30 seconds at 55°C, and 40 seconds at 72°C for 42 cycles. The qPCR was performed and analyzed using a Rotorgene qPCR machine (Qiagen, Valencia, CA). Gene expression in each sample was normalized to the expression of B2MG and compared to the DMEM group using the \(2^{-\Delta\Delta T}\) CT method as follows:

\[
\Delta\Delta C_t = (C_t \text{ Target gene} - C_t \text{ Reference gene}) \text{ Experimental} - (C_t \text{ Target gene} - C_t \text{ Reference gene}) \text{ Control}
\]

5.2.9 Protein isolation

A separate set of cell culture plates was used for quantification of elastin deposited in the cultures and in the medium. At the determined time points as stated above, the medium was collected from cultures and stored. Cells were washed with PBS, and 1 ml of 1% Triton 100X (ThermoFisher Scientific, Waltham, MA) in PBS was added to each well. The well plates were sonicated for 30 minutes to lyse the cells and then the cell layer was scraped off using a rubber policeman. These lysed cells were centrifuged at 10000 rpm for 10 minutes to collect the supernatant, which contained the lysate protein. The remaining cell pellet was used to quantify matrix elastin. Protein from both the lysate and the medium collected was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) as per the manufactures protocol.

5.2.10 Gelatin zymography and Reverse zymography

Active MMP activity was analyzed in the medium collected from cell cultures using gelatin zymography. Resolving (10% polyacrylamide with 1 mg/ml gelatin) and stacking
(4% polyacrylamide with 1% sodium dodecyl sulphate (SDS)) gel solutions were prepared fresh for every assay. 10% ammonium persulfate and TEMED (Thermo Fisher Scientific, Waltham, MA) were added to polymerize the gels, and they were cast using Surecast (Thermo Fisher Scientific, Waltham, MA) gel casting equipment. For all samples, 125μg of total protein was loaded per well along with pre-stained molecular weight standards (Precision Plus Protein Standard, Bio-Rad, Hercules, CA). Samples were electrophoresed for 90mins at 90V. After electrophoresis, the gels were washed in 2.5% Triton-PBS solution and then developed in buffer (50 mM Tris buffer, pH 7.5 containing 5 mM CaCl₂, 200 mM NaCl, and 0.02% brij-35) overnight at 37°C. Gels were then stained (Coomassie Blue 0.5%, methanol 40%, and glacial acetic acid 10% in DI water) and destained (methanol 40%, glacial acetic acid 10% in DI water) to visualize the active MMP bands using a Geldoc It® (UVP LLC, Upland, CA) imager. The bands were analyzed using GelQuant© software. Reverse zymography was performed in a similar fashion but with a different gel composition to visualize tissue inhibitor of metalloproteinases (TIMP) activity in the samples. 15% polyacrylamide gel was prepared with 1 mg/ml gelatin and supplemented with 20U of type II collagenase. A separate part of the gel with the same samples was incubated in development buffer along with PGG (20μg/ml) for both gelatin zymography and reverse zymography to observe if PGG can inhibit MMP and TIMP activity.

5.2.11 Reactive oxygen species (ROS) analysis

ROS in pulmonary fibroblasts was analyzed under aforementioned growth conditions using CellRox® deep red reagent, as per the manufacturer’s protocol. Briefly,
rat lung fibroblasts were seeded in well plates. They were allowed to attach and grow for 24 hours in just the growth medium. Cells were incubated for 48 hours in CSE, TNF-α, CSE+PGG, or TNF-α+PGG before adding CellRox® Deep Red reagent at a final concentration of 5µM, followed by incubation at 37°C for 30 minutes. Cells were then washed thrice with PBS, and DAPI stain was added to visualize cell nuclei. Cells were washed again and were fixed with 4% formaldehyde before proceeding to imaging.

5.2.12 Immunofluorescence imaging of elastin

A different set of cells grown under the aforementioned conditions was imaged for elastin deposition. At 7, 14, and 21 days, cell layers were washed twice with PBS and fixed in 4% formaldehyde for 15 minutes at room temperature. This was followed by a very brief exposure (10-15s) to 5% beta mercaptoethanol (Calbiochem®, San Diego, CA) and Guanidine HCl (Avantor Performance Materials, Center Valley, PA) solution to digest the microfibrils surrounding the elastin fibers [278]. We observed that exposure for longer than 60s digested the whole cell layer. Then cells were washed thrice with PBS and incubated with a 1% bovine serum albumin (BSA) (Seracare, Milford, MA) for blocking. FITC tagged anti-elastin antibody (Bioss Inc., Woburn, MA) was added to the cells at a concentration of 10µg/ml and incubated for 1 hour after which cells were washed thrice to remove any excess unbound antibody. DAPI (Thermo Fisher Scientific, Waltham, MA) was added to visualize cell nuclei, and after three washes, cells were mounted using aqueous mounting medium with anti-fading agents (Biomek Corp., Foster city, CA). Images were captured using a EVOS XL Imaging system (Thermo Fisher Scientific, Waltham, MA) with the same exposure levels for all samples.
5.3 Results

5.3.1 Pentagalloyl glucose (PGG) concentration characterization

PGG did not damage the cells when grown for 14 days. Cells grown in 2, 5 and 10 µg/ml concentrations of PGG did not show any significant reduction in cell viability as compared to their respective control group grown in DMSO (Figure 5-1). The FASTIN assay for elastin quantification showed that the 10 µg/ml concentration of PGG aided in achieving significantly higher matrix elastin (Figure 5-2).

Figure 5-1: Live/Dead assay of rat pulmonary fibroblasts grown for 1 week in different concentrations of PGG dissolved in DMSO. Cells with equivalent amount of DMSO served as control in this study. Green represents live cells and red represents dead cells. Scale bar=400µm.
5.3.2 Cigarette smoke extract (CSE) characterization

Live/Dead images of 1% and 5% CSE cell groups did not show any significant difference in cell viability. However, from 10% CSE onwards, cell death was evident as seen in both the Live/Dead and dsDNA quantification assays as shown in Figure 5-3. From this, we concluded that a final 5% solution of prepared stock CSE is apt for our cell culture studies.

Figure 5-2: Bar graph showing quantified matrix elastin after three weeks, from the cell culture of rat pulmonary fibroblasts grown in different PGG concentrations (n=3). The values obtained were normalized to DMSO group values in order to compare (dashed line). * represents significantly different from DMSO group.
Figure 5-3: Viability of cells grown in various concentrations of CSE (A) Live/Dead assay of rat pulmonary fibroblasts grown for 96 hours in different concentrations of cigarette smoke extract (CSE). Green represents live cells and red represents dead cells. Scale bar=400µm (B) Bar graph showing the cell number quantified using Picogreen dsDNA fluorimetric assay (n=3). * represents significantly different control group.
5.3.3 Relative expression of LOX, LOXL1 and ELN genes

Quantitative PCR analysis of mRNA obtained from all the groups showed that expression of all three genes was upregulated in all of the groups as compared to the DMEM group at week 1. The TNF-α+PGG group showed the highest LOX expression with 6-fold upregulation as compared to the DMEM group, while the other groups stand at 2-3 fold expression. The elastin gene was not upregulated in the TNF-α group, while the other groups showed a 4-6 fold increase. These expression levels returned to the same level as housekeeping gene expression by week 2. A comparison of expression levels at week 1 and week 2 shows that the PGG treated groups had higher expression of these genes compared to their respective controls as shown in Figures 5-4.
Figure 5-4: Bar graphs showing relative expression of LOX and elastin genes, LOX (A-B), LOXL1 (C-D) and ELN (E-F) genes in rat pulmonary fibroblasts treated with TNF-α, TNF-α+PGG, CSE and CSE+PGG. Dashed line represents control group value. # represents significantly different from control group.
5.3.4 Elastin quantification

As compared to the DMEM group, by week 2, tropoelastin in the medium was found to be higher in the TNF-α and TNF-α + PGG groups, while the CSE and CSE+PGG groups showed a decrease in tropoelastin levels. However, by week 3 neither of the above mentioned four groups showed an increase in tropoelastin suggesting that the inflammatory conditions may be hampering tropoelastin production.

![Figure 5-5: Bar charts showing elastin quantified in the spent medium (A-B) and cell culture matrix (C-D) of rat fibroblast cultures after weeks 2 and 3 treated with TNF-α, TNF-α+PGG, CSE and CSE+PGG. Dashed line represents control group value. # represents significantly different from control group. Other significant differences between specific groups are also shown separately.](image)

On the other hand, the PGG and TNF-α + PGG groups showed significantly increased matrix elastin deposition in cell cultures by week 2 and week 3, whereas the others remain lower as compared to the DMEM group. The CSE group shows the least
matrix elastin deposited at both weeks 2 and 3 without any increase in the amount. On the other hand, the CSE+PGG group shows a tremendous increase in matrix elastin deposition by week 3 suggesting the effect of PGG in elastin deposition. These trends are depicted in the bar graphs in Figure 5-5.

5.3.5 LOX activity

As shown in Figure 5-7, LOX activity measured at week 1 showed that the PGG and TNF-α + PGG groups have higher activity than the DMEM group, while the CSE group showed lower LOX activity than the DMEM group. Again, with the presence of PGG in the CSE+PGG group, LOX activity was on par with the DMEM group. The TNF-α and TNF-α + PGG groups showed similar LOX activity as that of the DMEM group, but the CSE seems to be impeding LOX activity. The significant difference between the CSE and CSE+PGG groups by week 2 clearly shows the role of CSE on LOX inhibition (Figure 5-6).

Figure 5-6: Bar charts showing LOX activity quantified in rat pulmonary fibroblasts treated with TNF-α, TNF-α+PGG, CSE and CSE+PGG. Dashed line represents control group value. # represents significantly different from control group. Other significant differences between specific groups are also shown separately.
5.3.6 Gelatin zymography and Reverse zymography

Gelatin zymography clearly allowed us to visualize MMP activity. The TNF-α and CSE groups showed substantial MMP activity, which was diminished in their respective PGG treated groups at weeks 1 and 2 (Figure 5-7). Densitometry analysis performed using GelQuant software allowed us to quantify the intensity of the bands. Medium samples from the CSE+PGG group had significantly lower band intensity compared to the CSE group at both weeks 1 and 2. On the other hand, the TNF-α+PGG group at week 1 had lower levels of MMP-9 activity (p=0.06), but at week 2, it was significantly lower than the TNF-α group.

A separate part of the gel with the same samples, incubated in development buffer along with PGG (20µg/ml) showed reduction in MMP-9 (92kDa) bands (data not shown).

Figure 5-7: Gelatin zymography images showing MMP 9 activity in cell culture medium treated with TNF-α and CSE, treated with PGG respectively (A and B). Densitometry quantification of MMP activity bands from gelatin zymography. Dashed line represents control group value. # represents significantly different from control group. Other significant differences between specific groups are also shown separately.
This shows that PGG can inhibit MMP activity in an extracellular environment. In reverse zymography, TIMP activity was analyzed, which showed that PGG treated cells have less TIMP activity (Figure 5-8). This might point towards the reduced requirement of TIMPs with PGG acting to inhibit MMP activity. These results show that PGG can effectively inhibit MMP activity, which could be of huge potential for preserving elastin from elastolysis.

![Figure 5-8: Reverse zymography images showing TIMP activity in cell culture medium treated with TNF-α and CSE, treated with PGG respectively.](image)

5.3.7 Immunofluorescence of elastin

We could observe significant elastin deposition in cell cultures by week 3. Immunofluorescence imaging of elastin deposited in the cell cultures depicted fibrous elastin deposited in the PGG treated group compared to the control DMEM group. The PGG treated group showed fibrous elastin deposition in the cell layers while the control group showed small and localized elastin deposits near the cells. Immunofluorescence studies reinforced the aforementioned quantitative elastin results. This supported our elastin quantification results that we obtained from the cell culture layers (Figure 5-9).
We observed a significant reduction in the ROS found in rat pulmonary fibroblasts in the TNF-α+PGG and CSE+PGG groups compared to the TNF-α and CSE groups. This clearly confirmed that PGG could keep ROS levels lower and thereby asserting the anti-oxidant property of PGG (Figure 5-10).

5.3.8 ROS analysis

We observed a significant reduction in the ROS found in rat pulmonary fibroblasts in the TNF-α+PGG and CSE+PGG groups compared to the TNF-α and CSE groups. This clearly confirmed that PGG could keep ROS levels lower and thereby asserting the anti-oxidant property of PGG (Figure 5-10).
5.4 Discussion

We have investigated the effects of PGG, a polyphenol and a derivative of tannic acid, on elastin deposition, MMP activity and ROS levels in an *in vitro* model using rat pulmonary fibroblasts. Fibroblasts are one of the key cells that make elastin and other extracellular matrix proteins[40]. Pulmonary fibroblasts are important in the pathology of COPD as they participate in ECM remodeling. There are *in vitro* models that have used...
pulmonary fibroblasts to study the disease [279, 280]. Various agents have been used to mimic the inflammatory conditions that exist in COPD, but cigarette smoke extract has emerged as a key model [281]. TNF-α has a very important role as a pro-inflammatory cytokine in lung pathophysiology [282, 283]. The main advantage of using CSE is that it correlates well with in vivo exposure. Soluble components of cigarette smoke pass through the mucus layer before reaching the epithelial cells of alveoli that remain distal to the airways. We have used both CSE and TNF-α to mimic the disease in cell culture. CSE is prepared by bubbling the smoke directly from cigarettes into warm PBS or cell growth medium. Usually this solution is considered to be 100% stock and is added to cell cultures to obtain different final concentrations. We bubbled smoke from 2 cigarettes (Marlboro 100s) in 10 ml of PBS. We have checked for cell viability of pulmonary fibroblasts grown in different conditions of CSE before deciding a 5% solution of the stock to be suitable for our experiments.

We have three important findings that are discussed here. First, we observed that PGG can significantly increase elastin deposition in the cell layer matrix. We have observed that both TNF-α and CSE treatment caused an increase in elastin mRNA expression at week 1. This can be related to increased elastin expression in COPD patients [24, 284], as an attempt by the lungs to repair the damaged elastin. We then quantified the elastin protein in the medium to see if the protein is produced in the same fashion as that of gene expression. The CSE group has less medium elastin than the control group. Cells supplemented with PGG showed more medium elastin production compared to their respective controls (TNF-α+PGG> TNF-α, CSE+PGG>CSE). By the end of week 3, this
elastin was quantified to see if it was deposited in the matrix. In a similar fashion to medium elastin, the TNF-α and CSE groups did not show any significantly higher elastin deposition in the matrix compared to the DMEM group. We believe that the higher amount of elastin deposited in the TNF-α +PGG and CSE+PGG groups, with low medium elastin found in these groups, shows the effect of PGG in precipitating elastin into the matrix. This phenomenon can be compared to that observed by Jimenez et al., with 1 µg/ml tannic acid and ellagic acid in dermal fibroblast cultures [285]. They have also observed lesser elastin degradation when elastin was combined with tannic acid. Precipitation of elastin with PGG has been shown in our previous studies confirming that PGG binds to elastin and coacervates it [199]. In a separate set of experiments, we have observed that treatment of cells with CSE (5% final concentration) for one week followed by PGG (10µg/ml) treatment for one week as a therapy increases elastin deposition (n=3 per group; p-value=0.1) compared to cells grown in DMEM after one week of growth in CSE (data not shown). Further proof of the action of PGG comes from our observation that LOX mRNA expression increased in cells treated with PGG, and its activity remained higher than respective non-PGG treated controls. CSE has been shown to inhibit cross-linking of tropoelastin molecules to form elastic fibers [286]. Li et al., point out in their review that LOX can be affected at many stages including mRNA expression [287]. However, we found an increase in LOX mRNA expression at week 1, which returned to the same expression level as housekeeping gene. We believe this may be because of the variety of cigarettes and the concentration of CSE we have chosen to add to the cell cultures. Gao et al., have shown that LOX mRNA transcription can be perturbed using cigarette smoke.
condensate, but not extract, at a concentration of 80 µg/ml or more [288]. Elastin degradation is an important step in the pathophysiological path of COPD. Elastin degradation results in elastin derived peptides (EDPs), which have been shown to be chemoattractants for monocytes. Sellami et al., have even shown that intra-tracheal injection of VGVAPG amino acid (one of the EDP sequences) causes emphysema in a murine model [62]. We have shown here that treatment of pulmonary fibroblasts with PGG increases elastin mRNA expression and increases elastin deposition in the matrix even with lower medium elastin production by cells exposed to CSE and CSE+PGG.

Our second observation is about MMP activity in medium collected from the cell cultures. We quantified enzyme activity using gelatin zymography. There was a decrease in MMP activity in the TNF-α +PGG and CSE+PGG groups compared to the TNF-α and CSE groups respectively. These results are in agreement with many other studies that reported decreased MMP activity after treatment with polyphenols [207, 289]. We observed decreased band intensity when incubated in a solution containing 20µg/ml PGG. This shows that PGG can inhibit MMP activity outside the cells as well. Another interesting result comes from reverse zymography. We believe that ours is the first study to show this result using PGG in pulmonary fibroblast cultures. Lindner et al., have shown a spike in MMP-9 expression (90 fold) in human pulmonary fibroblasts after 24 hours of exposure to 10 ng/ml of TNF-α [290]. On the other hand Ning et al., have observed that cigarette smoke stimulates MMP-2 activity in human fibroblast cultures [291]. However, we have only observed MMP-9 activity using zymography but not MMP-2 using zymography. This may be because of insufficient enzyme present in the medium to be detected or low expression.
We do not know if PGG decreases MMP-9 expression in pulmonary fibroblasts accounting for the decreased MMP activity seen in the medium from PGG treated groups. Another interesting observation is decreased TIMP activity in the cultures which had PGG added to them. This TIMP activity is not affected by incubation of the gel in development buffer, which contains PGG. This observation is supported by findings of Seifart et al., where they show that ATRA treatment reduces TIMP activity [292]. We also observed reduced TIMP activity in rats treated using doxycycline nanoparticles for four weeks (data not shown). This might point towards normalcy being restored in terms of TIMP activity, with MMP activity being kept in check by PGG treatment.

Our third observation pertains to the anti-oxidant property of PGG. Polyphenols are equipped with multifactorial properties that are beneficial in controlling inflammation. Oxidative stress is an important factor present in COPD, which is caused by cigarette smoking in patients [293]. The anti-oxidant properties of polyphenols have already been documented [198, 294]. We saw a clear eradication of the signal from the fluorescent ROS substrate in cells supplemented with PGG along with TNF-α or CSE treatment. It is also interesting to note that this oxidative stress could have caused increased elastin expression in CSE and TNF-α groups of our experiment, as observed by Deslee et al., [295]. We believe this anti-oxidant property of PGG might help in controlling oxidative stress in COPD and thereby controlling damage in lungs. Finally, we also tested for collagen in cell cultures to investigate if PGG increases collagen deposition in the matrix. Finlay et al., [296] showed that collagen remodeling is a consistent feature of emphysematous lungs as supported by Mosquero et al.[297]. Increased collagen is seen as a process of repair by the
lungs. We found that PGG treatment does not cause any extra collagen creation either in soluble or insoluble forms (data not shown here).

5.5 Conclusion

In this research aim, we have shown that PGG treatment shows increased elastin production and deposition in rat pulmonary fibroblast cell cultures. Elastin damage has been shown to drive lung damage in emphysema. Thus, stabilizing and regenerating elastin can be a way to arrest the progression of this disease. Oxidative stress and MMP mediated connective tissue damage contribute towards the complexity of this disease. We have shown that PGG can suppress ROS levels and MMP activity in pulmonary fibroblasts significantly. We have also observed that PGG can increase elastin expression in pulmonary fibroblasts and helps form elastic fibers in the matrix. This shows PGG can be used as a multifunctional drug in the treatment of emphysema.
6 SPECIFIC AIM 4: TO INVESTIGATE WHETHER PGG LOADED NANOPARTICLES CAN BE TARGETED TO EMPHYSEMATOUS LUNGS AND WHETHER THEY INCREASE ELASTIN MATRIX DEPOSITION, THUS BRINGING BACK THE ELASTIC RECOIL OF LUNGS

6.1 Introduction

Emphysema is a condition present in patients with COPD. Its characteristic features are chronic inflammation, oxidative stress, elastin damage and progressive alveolar destruction [241]. Improvement in lung function does not occur even after smoking cessation [298]. Cigarette smoke insult triggers the inflammatory response in lungs and over a period of time recruitment of more inflammatory cells causes excessive release of pro-inflammatory mediators leading to a disruption of protease-antiprotease balance in the lungs. Cigarette smoke also disrupts cell proliferative mechanisms, causes apoptosis, and inhibits alveolar and elastin repair, which make the condition irreversible [65, 68, 80].

Elastin protein is very important to organs like the lungs as it provides elastic recoil for the ease of breathing. Elastin degradation has a significant impact on patients’ lung function, and it has received little attention as a potential target for treatment of emphysema. Loss of lung elasticity has been correlated to a loss of lung function in emphysema patients [61]. The inability of adults to regenerate elastin has been attributed to the lack of coordinated expression of all the molecules required in the process of synthesizing crosslinked elastin [46]. Moreover, EDPs act as chemo-attractants for monocytes and further increase the inflammatory burden on lungs [299].
Preservation of elastin from damaging elastolytic activity is an important step towards preventing emphysema progression. If the lost elastin in the lungs is restored, then it is possible to reverse the disease. Pentagalloyl glucose (PGG) is a polyphenol that has been shown to preserve elastin from elastolysis in vascular tissues. It has also been shown to regenerate elastin in abdominal aortic aneurysm animal models [218]. Thus, in this research aim we tried to use PGG as a possible therapy for reversing mild emphysema in mice. We investigated if nanoparticles can be delivered through inhalation and the extension of their retention in lungs. Furthermore, we also study if PGG loaded BSA nanoparticle delivery to lungs can preserve elastin from degradation.

6.2 Materials and methods

6.2.1 Ex vivo PGG treatment

Frozen rat lung and aorta pieces were cut, washed and lyophilized to record initial dry weights. One group of samples were incubated in 0.05% PGG in MES buffer 24 hours. The others were only treated in MES buffer for 15 minutes. Following this both control and PGG fixed samples were subjected to elastase challenge in 5U/ml porcine pancreatic elastase solution (supplemented with 100mM Tris, 1mM calcium chloride and 0.02% sodium azide; pH 7.8) for 24 hours. They were again lyophilized to measure final dry weight. Percentage weight loss was calculated in both groups (n=5 per group). A separate set of samples treated in the same way, without lyophilizing, were used for histological examination.
6.2.2 Elastase inhibition by PGG

In order to investigate PGG’s ability to inhibit elastase activity, enzymatic assay was performed using N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SucAla3-pNA) (Sigma-Aldrich, St. Louis, MO) as substrate. Originally described by Beith et al., [300] in this assay, elastase cleaves this substrate into N-Succinyl-Ala-Ala-Ala and p-nitroanilide. Porcine pancreatic elastase (2U) was added to 120-µl of the substrate (2mg/ml) with or without PGG. Absorbance was measured at 410nm and percentage inhibition was calculated as the difference compared to absorbance of uninhibited sample.

\[
\text{Elastase} \quad \text{SucAla}_3\text{-pNA} + \text{H}_2\text{O} \rightarrow \text{SucAla}_3 + \text{pNA}
\]

6.2.3 Preparation of DiR dye loaded BSA nanoparticles (DiR-BSA NPs)

DiR dye (PromoCell GmbH, Heidelberg, Germany) loaded BSA (Seracare, Milford, MA) nanoparticles were prepared using desolvation method and conjugated to anti-elastin antibody (US Biological, MA, USA) for targeting purposes described previously [217-220]. Briefly, 250 mg of BSA was dissolved in 4ml of DI water. 2.5 mg of DiR dye dissolved in acetone was added to BSA solution. The mixture was stirred for one hour at room temperature following the addition of glutaraldehyde (EM grade 70%, EMS, PA, USA) at a concentration of 42µg/mg BSA. The mixture was added dropwise to 24ml of ethanol while sonicating (Omni Ruptor 400 Ultrasonic Homogenizer, Omni International Inc, Kennesaw, GA). The mixture was sonicated on ice for 30 minutes. The DiR-BSA NPs were separated by centrifugation at 10,000 RPM for 10 minutes and washed with water by
resuspension. These nanoparticles were prepared fresh and conjugated the day before treatment to animals.

6.2.4 Tagging NPs with elastin antibody

DiR-BSA NPs were PEGylated (mPEG-NHS, PEG succinimidyl ester, MW 2000) (Nanocs, New York, NY) by incubating 2.5 mg of PEG with 10 mg of nanoparticles at room temperature for one hour. The elastin antibody (United States Biological, Swampscott, MA) was thiolated using Traut’s reagent. 1 mg/ml of Traut’s reagent prepared in HEPES buffer at pH 8.8. 10µg of elastin antibody was added to 34 µl of 1mg/ml Traut’s reagent and 400µl of HEPES buffer. The thiolation process was carried out at room temperature for 1 hour, followed by filtration through 30kDa MWCO filter at a centrifugation speed of 10000g for 1 min. Finally, the thiolated antibody and PEGylated NPs were combined and incubated on a rocker shaker overnight at 4°C, to obtain ELN-DiR-BSA NPs.

6.2.5 In vivo targeting of nanoparticles to mice lungs

Eight-week-old male C57BL/c mice were subjected to intra-tracheal instillation of porcine pancreatic elastase and allowed to develop lung damage over 4 weeks of time. There were three groups of mice- viz. saline, one PPE injection at the starting (PPE1), and two biweekly injections (PPE2) at the start of weeks 1 and 3 (n=4 per group). After 4 weeks, ELN-DiR-BSA NPs were injected at a dose of 10 mg/kg via tail vein. Twenty-four hours after injection, mice were euthanized to image lungs and other organs for DiR signal. After compliance measurement lungs, liver, aorta, kidneys and spleen were harvested to
check for DiR signal by imaging each organ with IVIS® Lumina XR Imaging system (Caliper Life Sciences, Waltham, MA) set to excitation/emission of 745nm/790nm. Background signal was subtracted before analyzing the signal intensities from the organs.

6.2.6 Nanoparticle delivery via inhalation

Six week old male C57BL/c mice (n=12) were used for this study. In an effort to cause uniform lung damage by elastase in both the lungs, animals were made to inhale elastase delivered by a nebulizer. Control animals received saline inhalations, while the elastase groups’ animals received one-time elastase inhalation of 13.67U/ml porcine pancreatic elastase solution (PPE) (Elastin Products Co., Owensville, MO), dissolved in phosphate buffered saline (PBS) and aerosolized using a nebulizer system (Braintree scientific, Braintree, MA). Mice were anesthetized using 2% isofluorane and placed in a 12-pie cage for inhalations (Figure 6-1). The amount of aerosolized liquid deposited over 15 minutes duration was determined before the experiment to optimize elastase and nanoparticle concentrations. Four weeks after elastase inhalation, when sufficient elastin degradation is demonstrated, ELN-DiR-BSA NPs were given either by inhalation (ELN-DiR-Inh) route (n=3) (1mg/ml solution nebulized for 15 minutes) or via tail vein injection (ELN-DiR-IV) (n=3) (10mg/kg). Three days after administering nanoparticles, mice were euthanized and lungs were imaged as described before. In another study, one dose of either ELN-DiR-BSA NPs (ELN-DiR-6wk) or DiR-BSA NPs (DiR-6wk) (n=3 per group) were
delivered via inhalation after 3 days of damage and the animals were euthanized after 6 weeks to observe retention of both particles in lungs (Figure 6-2).

Figure 6-1: Nebulizer and pie cage set up for inhalation delivery of solutions.

Figure 6-2: Timeline graph of animal study to deliver DiR-BSA NPs via inhalation and intravenous injections in mice.
6.2.7 Preparation of PGG loaded BSA NPs

PGG-loaded BSA nanoparticles were prepared as described by Nosoudi et al., [204]. Briefly, 250 mg of BSA was dissolved (Seracare, MA) in 4 ml of deionized water. Pentagalloyl glucose (PGG, 125 mg) was dissolved in 200µl of dimethyl sulfoxide and added slowly to the BSA solution. After an hour of stirring, the mixture was added dropwise to 24 ml of ethanol under continuous sonication on ice for half an hour. Glutaraldehyde was added during stirring at a concentration of 12µg/mg protein (BSA). The elastin antibody conjugation procedure was similar to that of DiR-BSA NPs.

6.2.8 Targeted delivery of PGG-BSA NPs in vivo

Six week old male C57BL/c mice were divided into four groups i.e., healthy (n=6), non-treated (n=14), ELN-PGG-NP (n=6) and PGG-NP (n=6). As described above, healthy animals received saline inhalations while the other groups’ animals received one-time elastase inhalation of 25U/ml porcine pancreatic elastase solution (PPE) (Elastin Products Co., Owensville, MO), dissolved in PBS and aerosolized using a nebulizer system (Braintree scientific, Braintree, MA).

After inhalation, mice were kept for 5 weeks to allow lung damage. One week post elastase challenge, ELN-PGG-NP group received two biweekly inhalations of ELN-PGG-BSA NPs while PGG-NP group animals received similar biweekly inhalations of PGG-BSA NPs (without elastin antibody conjugation). 1mg/ml solution of freshly prepared
particles was nebulized for 15 minutes using the same equipment described above (Figure 6-3).

![Timeline graph of animal study to deliver PGG-BSA NPs in mice.](image)

**6.2.9 Measurement of air volume and mean lung volume intensity**

Mice were anesthetized and micro CT scans were performed on their lungs at 1 and 5 weeks post elastase challenge, using Skyscan 1176 micro CT (Bruker, Belgium). List mode scan with 12 frames was performed using the following parameters: voltage-50 kV, filter-Al 0.5 mm, current-500 µA, resolution-35 microns, exposure-50 ms with a rotation step of 0.7°. Images were reconstructed and analyzed using manufacturer provided software. After reconstructing the datasets, they were analyzed using CTAn software. Lungs were segmented out from other tissues and blood vessels using specific set of operations. 3D analysis of these datasets yielded the volume of air in the lungs at different phases of breathing. Absorption coefficient was calibrated with a water phantom to get accurate Hounsfield units value for the intensity of the segmented lungs. Using the
segmented out lungs as mask, mean intensity of the lung tissue was calculated from an intensity histogram (Figure 6-4).

**Figure 6-4**: Lung segmentation and mean lung volume intensity measurement using CT-An software.

### 6.2.10 Measurement of lung functional parameters

At the end of the study, mice were anesthetized using xylazine and ketamine to perform tracheotomy. Dosages for xylazine and ketamine were 5mg/kg (diluted 1:10) and 80mg/kg respectively. The animal was allowed to go into deep anesthesia so as to reduce the breathing rate. It was important to keep the animal in this state to avoid the animal fight the ventilator. An incision was made on throat and fascia was separated using forceps to
visualize trachea. Trachea was nicked and a connector tube was inserted into it. After tightly connecting the tube to trachea by a double knot suture, the animal was connected to FinePointe resistance and compliance system (DSI, St. Paul, MN) for measurements. The instrument had a ventilator that pumped known amounts of air and simultaneously measured an array of parameters such as lung dynamic compliance, resistance and tidal volume. The animals were euthanized by exsanguination under 4% isofluorane. Following the opening of chest cavity, whole body flush was performed by injecting heparinized saline in the right ventricle and cutting open the right atrium. This allowed both pulmonary and systemic circulatory vessels to be flushed. After the organs were perfused, one half of the lungs was frozen using liquid nitrogen for protein analysis while the other half was fixed in neutral buffered formalin. Other organs such as liver, spleen, kidneys were collected and fixed in neutral buffered formalin.

6.2.11 Measurement of MMP activity

Frozen lung pieces were homogenized in RIPA buffer (10 mM Tris-Cl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF; pH 8.0). After disrupting the tissue with a hand held homogenizer for 5 minutes, the samples were sonicated on ice for five more minutes to ensure complete homogenization. They were then spun at 10,000 rpm for 5 minutes and the supernatant was collected. MMP activity in the tissue homogenate samples was measured using internally quenched peptide substrates for MMPs 2&9 (Ex/Em= 280/360 nm, MMP Substrate III, Anaspec, CA) and MMP 12 (Ex/Em = 325/393 nm, 390 MMP FRET Substrate V, Anaspec, CA). One mg of the substrate was dissolved in 50 µl of DMSO, and the solution was diluted
in 10 ml of development buffer (50 mM Tris Base, 5 mM CaCl$_2$$\cdot$2H$_2$O, 200 mM NaCl, 0.02% brij 35). 2µl of the substrate stock solution and 2 µl of the extracted protein were mixed with 96µl of the development buffer and incubated for one hour at 37°C. A fluorescent plate reader was used to read endpoint florescence intensity.

6.2.12 Histology

Sections from formalin fixed lung pieces were assessed for damage in lungs. Processed tissue samples were embedded in paraffin and sections of 5µm thick were made from the sagittal face. H&E staining was used to observe general structural characteristics and enlargement of alveoli. VVG staining (Polysciences Inc., Warrinton, PA) was performed according to manufacturer’s protocol to study elastin damage in the alveolar walls of the tissue.

6.3 Results

6.3.1 Ex vivo PGG treatment

Weight loss after elastase challenge was determined in both aorta and lung samples to investigate if PGG can protect elastin from damage. PGG fixed samples showed significantly less weight loss after elastase challenge (Figure 6-5). This finding was also confirmed using Verhoff van Gieson staining of sections. Elastin was preserved in both lungs and aorta samples in PGG treated samples while it was degraded in the untreated samples after elastase challenge (Figure 6-5). FASTIN assay was performed on lung samples to quantify the amount of elastin per mg of dry tissue to further confirm the
stabilization of elastin. Concurring with the previous results, PGG fixed samples showed strikingly more elastin than control samples (Figure 6-6).

Figure 6-5: Results showing protection of elastin by PGG from elastase challenge ex vivo. A and B show percentage of weight loss in control and PGG fixed lung and aorta samples. C and D show representative histological sections of PGG fixed lungs and aorta where elastin is preserved. E and F show representative histological sections of control lung and aorta samples where elastin is depleted due to elastase challenge. Inset picture shows elastin preserved in PGG treated group versus elastin damaged in
6.3.2 Elastase inhibition by PGG

PGG inhibited elastase activity in a concentration dependent manner just after 20 minutes of treatment. Starting from 1µg/ml PGG concentration, elastase activity was inhibited by 40% compared to control samples. With a maximum concentration of 20µg/ml PGG inhibited up to 60% of PPE activity. This result suggests that PGG might also decrease elastase activity and therefore improves elastin stabilization further (Figure 6-7).
6.3.3 *In vivo* targeting of nanoparticles to mice lungs

IVIS imaging of mice lungs showed scintillating difference in the amount of fluorescence seen among the groups. Saline injected mice did not show any fluorescence signal while elastase damaged mice lungs showed significantly high amounts of fluorescence. Aortae from same animals, that were healthy did not show any signal, further confirming that nanoparticles only target degraded elastin (Figure 6-8). We noticed a decrease in the signal in the lungs of mice, which were given two doses of PPE. This might be because of excess elastin loss, which impacts the binding of nanoparticles. A plot of DiR signal versus elastin quantified from the mice lungs shows a negative correlation.
between these variables suggesting elastin damage is directly correlated for the nanoparticle signal (Figure 6-9).

Figure 6-8: Fluorescence from DiR NPs show successful targeting of anti-elastin antibody tagged nanoparticles to only lungs with elastin damage. Comparison of signal from aorta proves that these nanoparticles spare the healthy elastin in them.
6.3.4 Histology

Hematoxylin and Eosin staining of control, PPE1 and PPE2 group mice lung samples showed the extent of damage correlated with elastase treatment. The damage increased with the increase in the elastase dose. Verhoff van Gieson staining for elastin also indicated less elastin around the alveolar walls of PPE damaged mice lungs. Immunohistochemistry with elastin antibody (US Biological, Salem, MA) showed depleted elastin in the same areas giving us a model validation and targeting of our nanoparticles to damaged elastin (Figure 6-10).

6.3.5 Nanoparticle delivery via inhalation

With an aim of creating uniform but also a milder damage to lungs, mice were allowed to inhale elastase instead of intra-tracheal instillation. We compared dose delivered
by either inhalation or systemic route (Figure 6-11A). We observed less signal counts in the inhaled nanoparticle group compared to tail vein injected group clearly suggesting that systemic delivery was more effective in targeting lungs. Although the concentration of particles given via nebulizer was matched with that of tail vein injection dosage, the whole body inhalation setting might have affected the amount of particles inhaled and therefore caused less signal in inhalation group. Next, we investigated if elastin antibody conjugated nanoparticles stay in the lungs for a longer time compared to unconjugated inhaled particles; animals were allowed to survive for 6 weeks after administering nanoparticles. We observed that even after 6 weeks, elastin conjugated particles persisted in the lungs with just one inhalation (Figure 6-11B).
<table>
<thead>
<tr>
<th>Saline</th>
<th>PPE1 (One elastase injection)</th>
<th>PPE2 (Two elastase injections)</th>
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Figure 6-10: Histological analysis of lung sections from control, PPE1 and PPE2 groups (left to right) of mice. A-C show Hematoxylin and Eosin staining depicting lung tissue damage (Scale bar=50µm). D-F show Verhoff van Gieson (VVG) staining depicting loss of elastin from the alveolar septae (Scale bar=20µm). G-I show immunohistochemistry which again depicts loss of elastin along the alveolar walls. (Scale bar=50µm).
Figure 6-11: Fluorescence images of lungs showing nanoparticle targeting via different routes (A) Fluorescence from ELN-DiR-BSA NPs show successful targeting of anti-elastin antibody tagged nanoparticles to only lungs with elastin damage via both intravenous and inhalation routes of delivery. (B) Nanoparticles conjugated with elastin antibody (ELN-DiR-BSA NPs) are found in mice lungs after 6 weeks of inhalation while unconjugated nanoparticles (DiR-BSA NPs) are cleared.
6.3.6 Measurement of air volume and mean lung volume intensity

Average volume of air in the lungs was measured at weeks 1 and 5 for all mice. We did not notice any significant change in this volume between 1 and 5 weeks (all the groups showed about 250 mm$^3$ of air in their lungs) (Figure 6-12A). The mean lung volume intensity, which quantifies the damage of tissue, has also remained same in all the groups without any statistically significant difference (Figure 6-12B). This data clearly suggests that only milder emphysema was induced with the elastase treatment, which was enough to test the loss of elastin without changing the lung functional parameters drastically.

![Figure 6-12: Bar charts showing lung volume parameters measured using micro CT](image)

- **A**: Bar chart showing average volume of air in the lungs of all mice groups in the study, measured at weeks 1 and 5 post elastase challenge.
- **B**: Bar chart showing mean lung volume intensity values (HU units) of all mice groups in the study, measured at weeks 1 and 5 post elastase challenge.
6.3.7 Measurement of lung functional parameters

The most important finding of this study came from observing the compliance and resistance of the lungs after elastase inhalation. We noticed a small dynamic compliance increase in elastase treated mice (n=12) compared to the healthy subjects (n=6) (0.03 vs 0.024 ml/cm H$_2$O; p<0.05). When, PGG loaded nanoparticles were delivered after elastase treatment, we found that compliance was restored in ELN-PGG-NPs group of mice (n=6) (0.025 ml/cm H$_2$O) but not PGG-NPs (n=5) (0.03 ml/cm H$_2$O) (Figure 6-13). Similarly, lung resistance was lower elastase treated mice compared to control healthy mice (1.75 vs 1.108 cm H$_2$O/ml/s). Lung resistance in ELN-PGG-NPs group was intermediary to control healthy mice and elastase-treated groups (1.35 cm H$_2$O/ml/s). PGG-NPs group had similar reduced lung resistance value (0.91 cm H$_2$O/ml/s) as that of elastase-treated group (Figure 6-14). Falling on the same lines, tidal volume of healthy mice was the smallest, increased

![Figure 6-13: Bar chart showing dynamic lung compliance (C$_{dyn}$) values in all groups of mice. Differences between groups are shown specifically using lines.](image-url)
in elastase-treated group (138.5 vs 160.6 mm$^3$; p<0.05). ELN-PGG-NPs group showed a trend of improvement (145.9 mm$^3$) of tidal volume, while PGG-NPs group showed no sign of decrease in tidal volume (160.8 mm$^3$). These results indicate that only a mild emphysema was induced in mice lungs but with a damage to elastin (Figure 6-15) and PGG loaded and elastin antibody targeted nanoparticles did improve lung parameters.

Figure 6-14: Bar chart showing lung resistance values in all groups of mice.

Figure 6-15: Bar chart showing tidal volume values in all groups of mice. Differences between groups are shown specifically using lines.
6.3.8 Measurement of MMP activity

MMP 2, 9 and 12 activities were measured using specific FRET substrates. The group of mice, which were left untreated, had the highest amount of MMP activity per mg protein in their lungs. This activity came down in the groups treated with ELN-PGG-NPs and PGG-NPs. Control group of mice that were not treated with elastase (healthy) had the lowest MMP values and PGG groups also reduced MMP levels to almost similar levels (Figure 6-16). This shows that PGG nanoparticles can inhibit MMP activity in vivo.

Figure 6-16: Bar charts showing MMP 2&9 and 12 activities quantified in lung samples. Significant differences between groups are shown using lines.
6.3.9  Histology

Verhoff van Gieson staining for elastin indicated less elastin around the alveolar walls of non-treated group mice lungs (Figure 6-17 A-D). Control mice had intact elastin around the alveoli. ELN-PGG-NP group had elastin preserved along the alveoli compared to the diminished elastin in non-treated group. Immunohistochemistry with elastin antibody showed similar observation of depleted elastin in non-treatment group (Figure 6-17 E-H). In ELN-PGG-NP group, more elastin was present in the alveoli with preserved air spaces. PGG-NP group had intermediary elastin preservation as compared to ELN-PGG-NP group. Luna stain showed elastin fibers depleted from the alveolar walls of non-treated lungs, while ELN-PGG-NP group showed retention of elastic fibers (Figure 6-17 I-L).
Figure 6-17: Histological examination of mice lungs. A-D show Verhoff van Gieson (VVG) staining of control, non-treated, ELN-PGG-NPs and PGG-NPs group lungs. Loss of elastin fibers from the alveoli can be noticed in the non-treated group compared to control and ELN-PGG-NP group. E-H show immunohistochemistry of the above groups with anti-elastin antibody. Results depict elastin preserved in ELN-PGG-NP group lungs while it can be seen eroded in the non-treated group and PGG-NP group. Final row shows luna staining of mice lung sections (I-L) which also shows preservation of elastin fibers along the alveolar walls (purple fibers) with ELN-PGG-BSA NPs treatment.
6.4 Discussion

Here we show that PGG loaded nanoparticles can be targeted to degraded elastin in the emphysematous lungs and do they prevent further loss of elastin in lungs and improve lung properties. Elastase model of emphysema was used to achieve elastin damage that led to changes in lung functional parameters. Elastase emphysema facilitates faster induction of disease as compared to smoke inhalation [301]. Although hamsters are the most susceptible to lung damage owing to their low alpha-1 antitrypsin levels [302], rats and mice offer a more convenient option to study this damage using elastase model. Between rats and mice, rat lungs are less susceptible to elastase injury [303]. Additionally, mice offer possibility of manipulation of genes (knockout and knock-in) so that the molecular mechanisms of disease progression and therapy can be studied. Therefore, we used a mouse model of emphysema for this research.

First we investigated if PGG can protect elastin in the lungs from elastase challenge. We could demonstrate elastin preservation in both lung and aorta tissues treated with PGG followed by elastase challenge \textit{ex vivo}. Tam et al., have shown similar result on preservation of native elastin in porcine aortic valve leaflets treated with a novel fixative containing PGG as one of its components [304]. Isenburg et al., have previously shown that periadventitial application of PGG on aorta hindered the development of abdominal aortic aneurysm in rats [276]. Nosoudi et al., have not only observed elastin preservation but also reported elastin regeneration after delivery of PGG loaded nanoparticles in a rat model of abdominal aortic aneurysm [305]. We observed significantly lower loss of weight after elastase treatment in PGG treated lungs. PGG is shown to bind to elastin and inhibit
elastase mediated degradation of elastin. Our observation of PGG also inhibiting PPE activity in vitro suggests that PGG might act dually, first by binding to elastin in lungs and protecting it from degradation, while free PGG also can bind to the PPE enzyme and make it less active.

Next, we demonstrated the reproducibility of our targeted nanoparticle delivery in mice by using elastase intra-tracheal instillations. Similar to our previous results with rats, we observed no fluorescence signal from lungs of control mice (saline instillation), whereas elastase challenged mice lungs showed significant signal corresponding to ELN-DiR-BSA NPs. Aortae, where healthy elastin was present, did not show any signal, thereby confirming that elastin antibody bound nanoparticles only target degraded elastin. We further show that ELN-DiR-BSA NPs remain in lungs 6 weeks after one-time nanoparticle inhalation. Thus, such nanoparticles can be used for prolonged drug delivery to the lungs.

Our elastase inhalation only caused mild emphysema in mice. There are several million patients are in the mild phase of emphysema and currently have no treatments to further prevent disease progression. According to a result published by Goossens et al.,[306] 47% of COPD patients are in mild stage of emphysema. According to a survey conducted by the National Health and Nutrition Examination Survey (NHANES) during 2007-2010, 20.1% of the US population was estimated to have COPD, with 10.9% and 7.8% were present in the mild and moderate stages of emphysema respectively [307]. According to a report published in 2011 by Fitch et al., number of patients with mild emphysema was 30% while 53% were grouped into moderate emphysema stage [308].This
shows that an increasing attention is required to stop the progression of disease to reduce the economic burden. Our studies with elastase inhalation allowed us to create elastin damage without decline in the lung function drastically so as to obliterate the tissue beyond repair. There are not many consistencies in the literature for extent of lung damage with elastase instillation. For example, Hamakawa et al., used 0.25 IU of PPE via intra-tracheal instillation and observed a change in compliance between control and PPE treated mice at 21 days (~0.02 vs 0.05ml/cm H\textsubscript{2}O) [105]. On the other hand, Szabari et al., used 6 IU of PPE, which is 24 times more than Hamakawa et al study and have observed similar damage after 21 days [111]. Takano et al. used 0.25 IU, 1 IU and 2 IU of PPE and have not observed any significant deviation in compliance from the control group of animals [309]. Cruz et al. have observed a decrease in the amount of elastic fibers in non-treated mice lungs compared to healthy controls (9.5 % vs 10.4%) [171]. Barrutia et al. have also used 6U of PPE to achieve significant damage to the lung parenchyma [310]. Following a more incisive approach, Vidal et al., have intubated mice for injecting 2U/100g body weight to observe very high damage to the lungs. In this context, our effort to induce a more uniformly distributed lung damage with a whole body inhalation of elastase has resulted only in change in lung compliance. Lung compliance is a parameter that depends on the elasticity of the tissue. Loss of elastin because of elastase activity has caused a slight but significant increase in the lung compliance in elastase-treated group (n=12) compared to healthy control group. When treated with ELN-PGG-BSA NPs after elastase treatment, lung compliance reduced to the same as that of control healthy group animals. These results clearly suggest NPs delivered PGG and inhibited elastase mediated damage to the lungs.
When treated with nanoparticles without elastin antibody conjugation (PGG-BSA NPs), we did not see change in lung compliance clearly suggesting that nanoparticles were cleared without an active targeting mechanism and were unable to retain and deliver PGG to the lungs. Elastin was preserved in ELN-PGG-NP group as seen in histological sections. Luna stain clearly demarcates elastin loss in non-treated and PGG-NP groups as opposed to ELN-PGG-NP group.

We have also observed a significant reduction in the MMP activity with our PGG nanoparticle therapy. This result is significant as MMPs have been known to drive the disease forward by degrading extracellular matrix components. A green tea polyphenol (-)-epigallocatechin 3-gallate has been shown to inhibit MMP2 secretion in glioblastoma cells [311]. With respect to emphysema, polyphenols like curcumin and xanthohumol have also been investigated for matrix metalloproteinase activity inhibition and even anti-inflammatory effects [209, 210]. Coupled with elastin preservation property of PGG, inhibition of elastase and MMP activity could be very beneficial in keeping lung damage in emphysema.

6.5 Conclusion

In conclusion, this research demonstrates the proof of concept for targeted delivery of nanoparticles in mice. We show that elastin antibody conjugation allowed the nanoparticles to remain in the lungs. PGG was shown to effectively preserve elastin in lung ex vivo, by binding to elastin as well as inhibiting elastase activity. A mild emphysema was created by elastase inhalations to achieve elastin damage in lungs in mice. Delivery of PGG NPs to
mild emphysema mice model protected elastin from degradation and retained lung compliance the same as control healthy animals. This suggests PGG, when delivered locally by targeted nanoparticles, has the potential to stabilize and regenerate elastin. Coupled with its anti-oxidant and elastase inhibition properties it can halt the progression of mild emphysema.
7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Elastin is an essential protein for maintaining elasticity of lungs and cells cannot repair elastin that is degraded due to inflammation in lungs in emphysema. Elastin degradation products are also capable of driving the inflammatory cycle by acting as chemokines for attracting inflammatory cells from blood stream. Treatments have so far focused on stopping inflammation by anti-inflammatory drugs, but such drugs cannot repair lost elastin in lungs. This permanent loss of elastin seriously affects the normal functioning of lung, even with subsided inflammation. Therefore, protecting elastin from degeneration and helping cells deposit mature elastin in the lungs can help in bringing back the elastic recoil and reverse the disease. With this aim in mind, we have developed unique albumin based nanoparticles that can target damaged elastin in the lungs. We showed that the anti-elastin antibody conjugation to these nanoparticles makes them stick damaged elastin in lungs for up to 6 weeks. After successfully demonstrating this targeting, we delivered doxycycline-loaded particles to the lungs. We showed that one dose of our nanoparticles loaded with doxycycline inhibited the MMP activity for 4 weeks. We further show that PGG can preserve elastin and inhibit elastase activity in an *ex vivo* setting. Further research was carried out to test if PGG delivered with NPs can target emphysematous lungs and protect it from progression of the disease. Animals treated with PGG nanoparticle showed more elastin in the lungs than the non-treated controls. MMP 2, 9 and 12 activities were suppressed when treated with PGG nanoparticle therapy and lung compliance remained same as that of control healthy animals. With these initial promising
results, we hope to take PGG treatment forward to develop it as a reliable therapeutic for emphysema treatment at an early stage of the disease.

7.2 Recommendation for future work

I. Our *in vitro* and *in vivo* data from this research shows that PGG can reduce MMP activity. It would be interesting to investigate the mechanism of this to gain further insight on the role of PGG. It will be useful to observe if this inhibition is due to impeded production of MMPs at cellular level or an extracellular inhibition of their activity. This can be done by following the same procedure as described in this dissertation, by culturing rat pulmonary fibroblasts and evaluating expression levels of MMPs with and without PGG treatment.

II. In this dissertation, we show the beneficial properties of PGG such as antioxidant property, MMP inhibition, and elastin deposition in rat pulmonary fibroblasts, under two types of inflammatory conditions to mimic emphysema. This approach can be extended to other types of cells like epithelial-fibroblast co-cultures, macrophage-fibroblast co-cultures and even human COPD subject pulmonary fibroblasts. This will provide us with more information as to how epithelial and macrophages, when subjected to inflammatory insult, interact with fibroblasts by producing various types of cytokines and whether PGG treatment can ameliorate these changes *in vitro*.

III. In this dissertation, we used the elastase model of emphysema. We have experimented with various dosages and routes of delivery of elastase to
induce damage. By inhaling elastase, we have observed a uniform damage of lung but this only created a mild damage. While PGG is able to protect the lung tissue with this amount of damage, it needs to be tested under conditions that are more aggressive. It would be useful to also test this in other animal models like cigarette smoke exposure, which produces more clinically relevant pathological changes compared to human emphysema. More information can be obtained by analyzing expression levels of inflammatory cytokines in the lung and remodeling extracellular matrix proteins.

IV. It would be interesting to examine the role of alveolar macrophages in an animal model of emphysema. Alveolar macrophages are key players in the progression of this disease and whether PGG can influence their role is important in determining its mode of action.

V. Pulmonary function tests are now used to diagnose COPD in patients currently. Looking at the importance of elastin in this disease, it would be very useful to develop a diagnostic tool using computed tomography (CT) and gold nanoparticle approach. With this novel elastin targeting in emphysematous lungs, gold nanoparticles can be targeted to damaged elastin in lungs. A quantitative relationship may be established to correlate the amount of nanoparticle binding and elastin damage in the lungs, giving us a way to detect elastin degradation and predict emphysema before it reaches to a moderate stage, where it becomes difficult to treat.
VI. We have shown that PGG assists in deposition of elastin in the matrix by rat pulmonary fibroblasts \textit{in vitro}. As cited in the literature, previous work in our lab has also shown that it binds to elastin to protect it from being degraded by elastases. More studies are needed to look at the mechanisms of how PGG aids elastin deposition. It will be very useful to find out if PGG is playing a catalyst role by holding tropoelastin molecules close to degraded elastic fibers and allowing lysyl oxidase to crosslink them and if PGG is actually consumed in this process. This analysis can be extended to other properties i.e., MMP inhibition and anti-oxidation.

VII. Finally, the targeting technique used in this research is a result of many years of successful testing in various animal models. To utilize this technique of targeting elastin damage in other lung disorders like fibrosis and cancer could be a way to deliver toxic drugs at significantly lower concentrations with a controlled release option. Additionally, nanoparticle surfaces could be modified to target known markers to specific cancers [312, 313].
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135


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