TARGETED DELIVERY OF ANTI-INFLAMMATORY AND ANTI-OXIDANT DRUGS FOR TREATMENT OF CHRONIC OBSTRUCTIVE PULMONARY DISEASES

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) was found to be the fourth leading cause of death in 2004, but current treatments can only relieve the symptoms and improve the quality of life for the patients. Glucocorticoids have been broadly applied in other inflammatory conditions with promising therapeutic effects, but performed poorly in COPD. The phenomenon called glucocorticoid resistance is responsible for glucocorticoid insensitivity in COPD. Researchers have proven that elevated oxidative stress in the lung of a COPD patient is one of the main causes of blocking the function of glucocorticoids. We hypothesize that reducing the oxidative stress in the lung can restore the therapeutic effect of glucocorticoids in COPD. Thus, simultaneous administration of antioxidant and glucocorticoid drugs could be a promising approach to treat this condition.

In order to achieve this goal, liposomes loaded with both glucocorticoids and antioxidants were developed and characterized. First, several types of nano-carrier candidates were prepared and tested, and based on the comparative study liposomes were selected as the nano-carrier system of choice for further experiments.

In order to quantitatively determine the relationship between the targeting efficacy and the amount of the targeting antibody immobilized on the surface of the liposomes, surface plasmon resonance (SPR) was performed. It was shown that initial increase of the affinity upon the increase
of the targeting antibody surface density was followed by the saturation of binding affinity at certain antibody surface density. This saturation surface density was independent regardless of the size of the liposomes or even the type of the targeting antibody and its ligand. This saturation point was important in optimization of the targeting efficacy of the liposomes coated with the targeting antibody.

In order to address the glucocorticoid resistance induced by the oxidative stress, and test the hypothesis of the synergistic action of the antioxidant and glucocorticoid drugs, a cell culture model was developed. Exogenous ROS were proven to be necessary in this model. The synergistic effect of the co-delivery of the two drugs was demonstrated by measuring pro-inflammatory response. Analysis of the gene expression of several important biomarkers showed that the mechanism of action involved restoration of the expression of histone deacetylases 2 (HDAC2) in the presence of the antioxidant. Later, this cell culture model also proved the increased drug availability in the case of targeted liposome-based delivery.

Overall, this targeted delivery system loaded both antioxidant and glucocorticoid drugs provided another approach to address the problem of treatment of COPD.
DEDICATION

I would like to dedicate this work to my family. My wife, Xinyi Wang, has been a constant source of support and encouragement during my graduate work. The dedication of my father Yuanfu Xiang, to his career as a senior engineer and to his family has inspired my research both professionally and personally, and my mother’s, Yimin Shi’s, pursuit of excellence in her career has also had a profoundly effect on me.
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I also offer my sincere gratitude to my collaborators from MUSC. Dr. Mark Kindy and his group worked with our group on the cerebral ischemia and reperfusion injury project. I also want to appreciate Dr. Yang Wei, who helped me with a series of surface plasmon resonance analysis. In addition, I would like to personally thank my colleagues from Nanobiomaterials Laboratory. I would never have finished my degree without the help of Dr. Vladimir Reukov, Dr. Gary Lee Thompson, Dr. Rohan Satishkumar, Dr. Victor Maximov, Dr. John Barry, Christopher Waddell, Chengyi Tu, and Raisa Kiseleva. The Bioengineering Department staff has been an instrumental source of support throughout my studies at Clemson.
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CHAPTER 1

INTRODUCTION

The goal of this study was to design nano-carriers loaded simultaneously with both antioxidant and anti-inflammatory drugs. Because the anti-inflammatory effect of glucocorticoids is hampered by oxidative stress, targeted delivery is required to achieve specific binding and prolonged residence time in the pulmonary system.

Chapter Two presents an overview of the background and clinical significance of this study. It includes a discussion of COPD pathogenesis, the structural alteration of a lung with this condition, its current treatments, and the direction of the research.

Chapter Three divides this work into 3 specific aims. First, this study focused on the formation and selection of the ideal nano-sized vehicle. In addition, surface plasmon resonance (SPR) was used to quantitatively investigate the targeting efficacy derived from the targeting antibody immobilized onto the nano-carriers. Finally, an in vitro model was used to investigate the anti-inflammatory effect of the co-delivery system.

Chapter Four discusses the various types of nano-carriers loaded with either antioxidants or glucocorticoids prepared using different mechanisms. Liposomes, double-emulsion poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs), and polylactic acid (PLA) NPs were
initially chosen as candidates. The properties of those nano-carriers, including size, zeta-potential, drug loading capability, and storage stability, were determined. Their stability was determined based on lyophilization with cryoprotectors and long-term storage at room temperature. Based on these analyses, liposomes were selected as the nano-sized vehicles for this study. The drug release profile of glucocorticoid was investigated through a dialysis-based technique with bovine serum albumin facilitating the release.

Chapter Five presents the analysis of the targeting efficacy derived from the targeting antibodies immobilized on the surface of the liposomes as determined by SPR in comparison to the conventional cell binding method. SPR is a more kinetic and quantitative technique for determining targeting efficacy than the cell binding method. This chapter covers the analysis of liposomes with various targeting antibody surface densities, sizes of liposomes, and types of the targeting antibodies. A pilot model of a secondary antibody targeting to its coupled primary antibody was conducted before testing the real targeting antibody. The avidity effect derived from the multiple binding sites on the surface of the liposome is also reported in this chapter; in addition, the saturation of the increasing trend of targeting efficacy derived from the avidity effect is discussed, an analysis essential for the further optimization of targeted liposomes.

The sixth chapter concentrates on the protective effect achieved from the liposome system using an in vitro model with glucocorticoid resistance. This model was developed by challenging
A549 cells with lipopolysaccharide (LPS), with an exogenous oxidant being used to achieve glucocorticoid resistance. To determine the cell response to the challenges and drugs, enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of released interleukin 8 (IL-8). In addition, quantitative SPR was used to analyze the gene expression of several important biomarkers relative to inflammatory response. In this chapter, the protective effect from the free drugs mixture and drug-loaded liposomes is presented.

Overall conclusions of the studies are included in Chapter Seven. First, targeted drug delivery devices carrying both antioxidant (SOD/SOD mimetic) and glucocorticoid (DEX) drugs were prepared and characterized. Second, the effect of the density of the targeting antibody on the affinity of nanoscale drug carriers was investigated, and optimal antibody density was determined. Third, the synergistic effect between glucocorticoid and antioxidant drugs was demonstrated in the A549 cell culture model.
CHAPTER 2

LITERATURE REVIEW

Chronic obstructive pulmonary disease (COPD) is a respiratory condition that involves chronic inflammatory of the lung tissue, usually caused by smoking. The term “COPD” was introduced in 2002 as a combined description of the two terms, “chronic bronchitis” and “emphysema” that were used previously [1]. According to the World Health Organization (WHO) assessment, COPD was the fourth leading cause of death worldwide in 2008. The incidence of COPD is projected to increase by more than 30% over the next 10 years, and COPD is expected to become the third leading cause of death by 2028. In 2013, COPD affected more than 24 million Americans, and has already become the third leading cause of death in the US. Disability caused by COPD significantly adds to the burden of this condition. At present, there is no practical cure as most of the current treatments are limited to relieving the symptoms, slowing its progress, and improving the quality of life for the patients.

2.1. Pathology of Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a disease induced by smoking, and its development and progress are mainly attributed to the increased oxidative stress from tobacco smoking. COPD can be categorized into three different pathological mechanisms which all cause
Chronic bronchitis is defined by cough and sputum, which are shown to be relative with airway mucosal surface, the submucosal glands, and gland ducts, particularly in the small bronchi between 2 and 4mm in diameter. This inflammatory results in an increase in airway smooth muscle, a generalized increase in the connective tissue in the airway wall, degenerative changes in the airway cartilage and a shift in the epithelial cell type.

Emphysema has been defined as “abnormal permanent enlargement of airspaces distal to terminal bronchioles, accompanied by destruction of the walls without obvious fibrosis”. Emphysema, caused by both the lack of elastic support in the alveolar attachment which disables the close of the small airways, and the decrease of intra-luminal pressure from loss of elastic recoil.

Airways obstruction in COPD is in the smaller conducting airways that include bronchi and bronchioles less than 2 mm diameter, caused by the destruction of alveolar support of the peripheral airways, the loss of elastic recoil in the parenchyma supporting the airways, structural narrowing of the airway lumen by the inflammatory remodeling process.

All these mechanisms will lead to air trapping and lung hyperinflation, resulting in dyspnea. FEV1 is the volume of air that can forcibly be blown out in one second, which is an important index to assess the lung function. For COPD patients, declined FEV1, or FEV1 over FVC (the volume of
air that can forcibly be blown out after full inspiration) is found, and these are the indicators to monitor the progression of COPD [3].

There are two direct consequences of reactive oxygen species (ROS) from tobacco use: epithelial injury and inflammatory response. Epithelial injury increases permeability of the respiratory epithelium right after the exposure to smoke, dramatically increasing the risk of infection, which plays a key role in COPD exacerbation. Up to two-thirds of exacerbations are related to microbial infection. Recent studies hypothesize the relationship between the infection and the risk of development of COPD. It has also been found that inflammatory is the essential mechanism of development of COPD [4].

2.1.1. Epithelial cells and macrophages

Oxidative stress is present whenever inflammatory exists. Studies have proven that for oxidative stress activates or enhance the inflammatory in COPD. Interleukin 8 (IL-8), an inflammatory mediator, which is usually indicative of the extent of inflammatory, is found to be released from epithelial cells upon increasing oxidative insult. Epithelial cells from COPD patients were found to release more IL-8 than those of healthy persons. 8-isoprostance and 4-HNE, produced by lipid peroxidation, can also act as signaling mediators to activate the release of inflammatory mediators such as IL-8 and Transforming growth factor beta (TGFβ). Studies hypothesized that the mechanism of smoke-induced inflammatory lies on a more fundamental level.
Redox-sensitive transcription factors have recently been shown to be increased by oxidative stress, such as (nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) [5]. NF-κB was found to play a key role in airway inflammatory, because many inflammatory mediators are regulated by NF-κB. It is present in cytosol as an inactive form, which is inhibited by protein (Inhibitor of κB) IκB [6]. Oxidants can result in activation of IκB kinase, cleaving IκB from NF-κB and releasing NF-κB. Studies from both macrophages and airway epithelial cells demonstrated that the release of inflammatory mediators, such as IL-8, IL-1, and NO correlate with the concentration of oxidants, which are also accompanied by enhanced nuclear binding and activation of NF-κB

Along with epithelial cells, alveolar macrophages play a critical role in the pathophysiology of COPD [7]. Normal macrophages are exposes to inhaled particulates, but produce low level of cytokines and are poorly phagocytic. This is because the cell-cell interaction induces TGF-β dependent expression of αvβ6 in alveolar epithelial cells, which suppresses both of the production of cytokines and phagocytosis [8]. Macrophages are activated by smoking due to release of inflammatory mediators.

2.1.2. Cytokines

Initially, CXC (proteins with two N-terminal cysteines which are separated by on an amino acid) and CC (proteins that have two adjacent cysteines) chemokines are immediately produced
after activation by alveolar macrophages. IL-8, common CXC cytokines as mentioned previously are important in the inflammatory network, and can respond to several stimuli, including smoke extract which contains a high concentration of reactive oxygen species (ROS). Alveolar macrophages from COPD patients were found to release much more IL-8 than in normal smokers and non-smokers. Along with other CXC chemokine including GCP-2/CXCL-6, GRO\(\alpha\)/CXCL1, GRO\(\beta\)/CXCL2, GRO\(\gamma\)/CXCL3 and NAP-2/CXCL7, IL8 has been found to be the major polymorphnuclear neutrophils (PMN) chemoattractant [9, 10]. Cytokines stimulate neutrophils via two different receptors namely CXCR1 and CXCR2. Enhanced levels of all of these chemokines are found in either COPD patients’ bronchoalveolar lavage (BAL) fluid or sputum compared with normal controls. Reversely, the neutrophils themselves may be a major source of CXCL 8 [11]. In addition, another group of chemokines also can be released from alveolar macrophages, including CXCL10 (IP-10), CXCL11 (I-TAC) and CXCL9 (Mig), which are found to be chemotactic for CD8\(^+\) TC1 and CD4\(^+\) Th1 cells via interaction with the CXCR3 receptor expressed on these cells [9]. Similarly, CXCL9, CXCL10, and CXCL11 are found to be increased in COPD. Moreover, since the CXCR3 ligands CXCL9, CXCL10, and CXCL11 are all induced by interferon gamma (IFN-\(\gamma\)), and (classification determinant) CD8\(^+\) T-cell are themselves a source of IFN-\(\gamma\), there is a high probability for further positive feedback. Another family of cytokines, which plays an important role in COPD is CC chemokines. Compared to CXC chemokines, CC cytokines induce the migration of monocytes, NK cells, dendritic cells, eosinophils and basophils, which all play
roles in attracting T-cells [12-14].

2.1.3. Lipid mediators

Besides cytokines, which are proteins, some bioactive lipid mediators are also important in the inflammatory response. These include leukotrienes (LTs), lipoxins (LX), prostaglandins (PG), and thromboxane (Tx). These can be generated by alveolar macrophages and other human lung cells, like mast cells, T-helper cells and other leukocytes, in inflammatory. Macrophages produce a substantial excess of leukotriene B\(_4\) (LT\(_B\)\(_4\)) with leukotriene C\(_4\) (LT\(_C\)\(_4\)) after stimulation. Also, it was found that in patients with COPD, LTB\(_4\) concentration was increased. The role of LTs in the pathophysiology of COPD is uncertain. LTB\(_4\) was found to be a potent neutrophil chemoattractant, and cysteinyl(cys)-LTs which cause bronchoconstriction and have found having proinflammatory effects, since it can promote the synthesis of tumor necrosis factor alpha (TNF-\(\alpha\)), IL-8, GRO\(\alpha\), CXCL1, and IL-6 by macrophage. prostaglandins D\(_2\) (PGD\(_2\)) is known as another essential lipid mediator. It is also known to generate chemotaxis of eosinophils and T\(_{H2}\) lymphocytes, and stimulate cytokine production by T\(_{H2}\) cells. Moreover, both PGD\(_2\) and PGF\(_{2\alpha}\) may play a role as a mediator of bronchoconstriction and airway hyperresponsiveness [15] [16].

2.1.4. Proteinases

Alveolar macrophages also secrete elastolytic enzymes. There are many proteolytic enzymes released in the lung, not only by macrophages, but neutrophils are also capable of degrading elastin.
They produce serine proteases, cysteine proteinase, and matrix metalloproteinases (MMPs) [17]. Serine proteases are characterized by a catalytic triad (His, Asp, and Ser) which forms a charge-relay system transferring of electrons from the carboxyl group of Asp to the oxygen of Ser, which later becomes a powerful nucleophile and is able to attack the carbonyl carbon atom in peptides. Neutrophil elastase (NE), cathepsin G, and proteinase 3 are common serine proteinase in COPD, which are mainly coming from neutrophils. On the other hand, another group of proteolytic enzymes, cysteine proteinases utilizes a sulfhydryl group of Cys as the nucleophile and is mainly produced by alveolar macrophages in COPD. Besides, MMPs is another group of proteolytic enzymes which have been implicated in COPD. Among them, MMP-2, MMP-9, and MMP-12 are well studied [18]. It has been proven that all these bioactive molecules are regulated by NF-κB, which is already proven to be activated by ROS in the lung. It has been found that these elastolytic proteins, especially MMP-12 and neutrophil NE, contribute to lung destruction via the elastin degradation, which is related to emphysema. By genetic engineering, MMP-12$^{-/-}$ and neutrophil NE$^{-/-}$ mice were significantly protected from cigarette induced emphysema [19]. Thus, Shapiro, S.D., et al. demonstrated a direct role for neutrophil elastase in emphysema and emphasized the interdependence of the proteinase and inflammatory cells in response to smoking. The degradation of elastin would introduce positive feedback to COPD, since the fragment of elastin has been proven to be an auto-immune target.
2.1.5. ROS signaling in COPD

Environmental and inflammatory cell-derived ROS have been implicated in the activation of transcription factors such as NF-κB and AP-1, and in the signal transduction and gene expression involved in cellular pro-inflammatory actions. NF-κB has been proven to switch on multiple inflammatory genes resulting in amplification of the inflammatory response [20]. The pathway of NF-κB activation is not totally clear, but there are several redox-sensitive steps in the activation pathway as mentioned above. In inflammatory, neutrophils and other inflammatory cells are attracted into the lungs. These cells can generate ROS via the NADPH oxidase system. Moreover, cigarette smoking is associated with an increase in myeloperoxidase (MPO) content in neutrophils, and MPO activity is found to be negatively correlated with FEV1 in patients with COPD. Also, increased amount of ROS including O$_2^•$ and H$_2$O$_2$ is found from smokers BAL fluid. Thus, the ROS from cigarettes can raise the inflammatory reaction in respiratory space via NF-κB and other signal transduction. Later, inflammatory cells can continuously generate secondary ROS in the lung providing a positive feedback for ROS in COPD.

2.1.6. Growth factors

The tissue repair process is supposed to return the tissue function back to normal, but in chronic tissue injury, like smoking, it appears that the repair process loses many control mechanisms and later the remodeling of tissue alters the normal function. Remodeling at the
alveolar level and fibrosis of the small airway are commonly found in COPD [4]. Many growth
tactors from macrophages, epithelial cells, and other inflammatory cells can influence the
phenotype of the tissue cells and induce tissue remodeling. Many cytokines and growth factors
can be found at the site of tissue remodeling, but it is hard to determine the causal relationship
between them. It is believed that TGF-β plays an important role in peribronchiolar fibrosis.
Among 5 isoforms, TGF-β1, is most abundant and well characterized. TGF-β can stimulate
extracellular matrix (ECM) production including collagen and fibronectin and reduce the
degradation of collagen [21]. It also can induce the transformation of fibroblasts into
myofibroblasts, which are contractile cells and produce more matrix proteins. However, matrix
protein production is potentially a pathogenic process for fibrosis. TGF-β can be up-regulated by,
IL-1, GM-CSF, PDGF, and TGF-β itself and it can also induce the secretion of FGE, PDGF, and
CTGF.

Other growth factors also play important roles. For example, VEGF, which is a major
physiological regulator of vascular growth, can cause hypoxic pulmonary vasoconstriction in
COPD. Cigarette smoking activates TGF-α and then activates EGFR, resulting in the increase of
mucus secretion.

2.1.7. Other inflammatory cells in COPD

Increased numbers of neutrophils are found in COPD patients’ BAL and sputum. The
recruitment of neutrophils is induced by many kinds of chemokines, including LTB₄, CXCL8, CXCL1 and CXCL5, which can be produced by alveolar macrophages, T-cells and epithelial cells, and even the neutrophils themselves, especially in the case of CXCL8. Besides the production of CXCL8, another obvious role of neutrophils is secretion of proteinases, which mainly are serine proteinase and matrix metalloproteinases.

Dendritic cells play a central role in the innate and adaptive immune response [22]. In COPD, they have been found to activate many kinds of cells including macrophages, neutrophils, T and B lymphocytes. T-lymphocytes are found to increase in the airway of patients with COPD. CD8⁺ cells (cytotoxic T cells) and CD4⁺ cells (mature helper T cells) are important in the inflammatory response in the airways. In general, the expression of CXCL10 attracts these T cells, which preferentially express CXCR3. The chronic colonization by bacterial and viral pathogens is one possible reason for T cell accumulation. Besides, injury directly from smoking or from inflammatory may uncover the antoantigens which are sequestered in intact tissue, the role of T cells in COPD is not clear, but it can definitely produce the damage in the lung. The T cells release perforins, granzyme B, and TNF-α, which can cause cytolysis and apoptosis of alveolar epithelial cells. These damages will release more antigenic material. Moreover, CD8⁺ cells can release cytokines that can enhance the inflammatory reaction. T-helper cells are the main source of Interferon-gamma (IFN-γ), which is a cytokine critical for immunity [9].
Eosinophil infiltration is found in COPD, especially in exacerbation of COPD. Since the exacerbation may relate to viral infection, the eosinophils have a role in antiviral defense.

### 2.1.8. Summary

As factors illustrated above, all the cytokines, inflammatory cells, lipid mediators, ROS, and growth factors form a general network of COPD pathogenesis (Figure 1) [23, 24]. Each factor is induced by others and also can cause the activation of many other factors. Actually, the causal relationship cannot be totally clarified. Many positive feedback mechanisms are involved in the process of COPD development, which is supposed to exaggerate the situation. Generally, irritants (mainly ROS) from smoke activate epithelial cells and macrophages to release chemotactic factors that attract inflammatory cells to the lungs. Some of the cytokines attract monocytes, and some attract neutrophils and monocytes, and others attract T-cells. All these inflammatory cells together with macrophages and epithelial cells can release protease which can degrade elastin leading to emphysema. The growth factors from epithelial cells and macrophages stimulate fibroblast proliferation resulting in fibrosis in small airways. Growth factors can also induce mucus hypersecretion. The growth factors, cytokines, and ROS can further amplify inflammatory via positive feedbacks loops.
2.2. Current pharmacotherapy of COPD

Due to the increasing mortality from COPD, there is a pressing need for effective drug treatment. Some treatments are non-drug therapies including dietary, smoke cessation, long-term oxygen therapy, mechanical bronchial ventilation, and certain surgical procedures. On the other hand, pharmaceutical therapy is also a reliable method of COPD treatment. Many studies have been done on a variety of potential therapeutic drugs, including bronchodilators, mediator antagonists, chemokine receptor antagonists, anti-inflammatory reagent, and anti-oxidant drugs.

Figure 1. Inflammatory cells and mediators involved in COPD. Inhaled cigarette smoke and other irritants activate epithelial cells and macrophages to release several chemotactic factors that attract inflammatory cells to the lungs.
Table 1. Potential therapeutic options in smokers with COPD

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking Cessation</td>
<td>Nicotine Replacement Therapy</td>
</tr>
<tr>
<td></td>
<td>Bupropion</td>
</tr>
<tr>
<td></td>
<td>Varenicline</td>
</tr>
<tr>
<td>ICS, ICS/LABA combination</td>
<td>Fluticasone/Salmeterol</td>
</tr>
<tr>
<td></td>
<td>Budesonide/Formoterol</td>
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<tr>
<td></td>
<td>Mometasone/Formoterol</td>
</tr>
<tr>
<td>Long-acting Anticholinergics</td>
<td>Tiotropium</td>
</tr>
<tr>
<td>Leukotriene Modifiers</td>
<td>Montelukast</td>
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<tr>
<td></td>
<td>Zileuton</td>
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<tr>
<td>HDAC2 Enhancers</td>
<td>Theophylline</td>
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<tr>
<td></td>
<td>Peroxynitrite Scavenger Drugs</td>
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<tr>
<td></td>
<td>Macrolides</td>
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<tr>
<td>Peroxisome Proliferator-Activated Receptor Agonists</td>
<td>Rosiglitazone</td>
</tr>
<tr>
<td>Other Anti-Inflammatory Agents</td>
<td>PDE4 Inhibitors</td>
</tr>
<tr>
<td></td>
<td>P38 MAPK inhibitor</td>
</tr>
<tr>
<td></td>
<td>TNF – α inhibitors</td>
</tr>
</tbody>
</table>

* ICS = Inhaled corticosteroids, LABA = Long-acting beta2-agonists, HDAC2 = Histone deacetylase 2.
2.2.1. Corticosteroids

Corticosteroids also known as glucocorticosteroids, are a traditional anti-inflammatory drug and are effective in controlling inflammatory conditions such as asthma and arthritis [26-32]. The inflammatory signals like TNF-α activate NF-κB indirectly inducing acetylation of histones by histone acetyltransferase (HAT), increasing the expression of genes encoding inflammatory proteins [27]. Corticosteroids can reverse this histone acetylation by activating histone deacetylase-2 (HDAC2). HDAC is family of enzyme which counteracts the function of HAT, which silence the expression of genes and HDAC2 was proven to play a significant role in silencing inflammatory genes.

Even inhaled corticosteroids have been proven effective in asthma treatment, and also been applied on COPD treatment for a long time. Its therapeutic effect became more controversial recently. One of the main concerns about this controversy is the concept of glucocorticoid resistance in the COPD patients. In the situation of COPD, relatively higher dosage of inhaled corticosteroid induced limited efficacy and significant adverse effect [33]. Many studies proposed diverse mechanisms of his phenomenon, and one popular theory is that the high exogenous ROS from tobacco smoking can either decrease the production of HDAC2 or reduce its activity [27], significantly compromised the suppression of inflammatory from corticosteroid.
2.2.2. Beta2-agonists

β2-agonists are traditional bronchodilators, which have been used for pulmonary diseases for a long time. Initially targeting asthma. β2-agonists can bind to the β2-adreniceptor presented on vascular smooth muscle cells leading to the relaxation of the airway smooth muscle [34]. The β2-adreniceptor is found to be a G-protein coupled receptor, whose α-subunits can get released by a β2-agonist binding. This subunit can stimulate adenylyl cyclase, which produce cyclic AMP (cAMP) from ATP. cAMP, known as an important secondary messenger of intracellular signal transduction, can activate protein kinase A. Protein kinase A later phosphorylates key targets which can change the phenotype of airway smooth muscle cells and relax the airway smooth muscle.

β2-agonists can bind to 3 α-helices from the transmembrane domain of receptors. However, some long-acting agonists like salbutaline and terbutaline can prolong the duration of action due to an additional lipophilic tail binding. Moreover, the substitution and reposition of the hydroxyl group of the catecholamine structure of β2-agonists are found to decrease therapeutic ability but increase the resistance to catechol-O-methyltransferase (COMT), such as salbutamol and terbutaline. A significant volume of published evidence supports the role of β2-adreniceptor in the treatment of COPD [35]. Physiologically, β2-agonists dilate the airways and reduce the air trapping and wheezing to improve lung function and improve the exercise tolerance, and thus improving the quality of life.
Later, some clinical benefit was determined from the combination of β₂-agonists with other potential reagents including antimuscarinic agents. Meanwhile, the combination of long acting β₂-agonists and corticosteroids has shown benefits in clinical studies. Many evidences demonstrated that these two agents have a synergy when combined. Cave et al. summarized that corticosteroids can increase the β-receptor density, and enhance Gαx expression. Reversely the β₂-agonists can recruit histone deaceylases to the promotor regions of target pro-inflammatory genes to repress transcription [36]. However, this combination also got compromised by the smoke induced HDAC2 reduction [37].

β₂-agonists are central in the symptomatic management of asthma and COPD. However, with its side effects and limited improvement of the lung function this treatment does not address the underlying mechanisms of COPD [38]. After long-term and high-dosage exposure some side effects become obvious and severe. Some studies indicate that β₂-agonists demonstrate pro-inflammatory effects [39, 40]. Salpeter et al. claimed that regularly inhaled beta-agonists increased the risk of respiratory death by more than two-fold, compared with a placebo [41].

Other bronchodilators are also applied to reduce COPD. Tiotropium bromide is one of anticholinergic bronchodilators, which is a muscarinic receptor antagonist that cuts off the cholinergic reflex bronchoconstriction. Moreover, some other bronchodilators such as Viozan are currently in clinical development. Viozan is a dual agonist of both β₂-adrenergic and dopamine D₂
receptor, which can inhibit cough and reflex mucus secretion.

2.3. Source and mechanisms of reactive oxygen species

As discussed above, ROS are strongly involved in pathophysiology of COPD. Furthermore, the most straightforward treatment option by glucocorticoids is also strongly affected and eventually rendered inefficient by ROS. Therefore, it is important to understand details of production and mechanism of action of ROS, which is discussed in the following section”.

2.3.1. Endogenous generation of ROS

ROS (Reactive oxygen species) are oxygen compound derived from metabolism, which includes superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH), peroxyl radical (ROO$^.$), alkoxyl radical (RO$^.$), ozone (O$_3$), and singlet oxygen (O$_2^*$) [42-45] (Figure 2). The formation of singlet oxygen can later result in the sequential reduction of superoxide, hydrogen peroxide, and hydroxyl radical. However, other research claim that O$_2^-$ is known as the precursor of most ROS in the cell, and this radical processes the most high oxidizability [45]. The cellular sources of ROS are mitochondria, cytochrome P450 family protein, xanthine oxidoreductase (XO), cyclooxygenases (COX), lipoxygenases, and the NADPH oxidase (Nox) family [46].
Figure 2. Generation of different ROS by energy transfer or sequential univalent reduction of ground states triplet oxygen.

Under normal metabolism condition, the main source of $O_2^-$ is the mitochondrial electron transports chain which is the major source of ATP in the body. About 1 to 3 percentage of $O_2$ can be reduced in mitochondria into $O_2^-$ by the electrons leaked from the chain. This superoxide can be produced by both complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase) of that electron chain. It also has been detected that $O_2^-$ from complex III is extra-mitochondrially release into cytosol directly.

In ischemia reperfusion, xanthine oxidase in cytosol would transfer electrons abnormally to $O_2$, rather than to $NAD^+ [47]$. Moreover, in the inflammatory, NADPH oxidase of phagocytic cells becomes the major source of $O_2^-$ in the tissue; these superoxide ions are important to kill any invading microorganisms. In addition, certain enzymes produce $O_2^-$ as part of their normal function. For example cytochrome, nitric oxide synthases, and cyclooxygenase can all produce $O_2^-$ as a side product. Furthermore, some bio-active molecules, such as glyceraldehyde, FMNH2, and
some thiol containing molecules (e.g., cysteine) can produce O$_2^-$ through auto-oxidation[45].

2.3.2. Oxidative damage

Traditionally, researches are focused on the oxidative damage induced by ROS through lipid peroxidation on membranes, DNA damage, and protein oxidation [48]. The most important of these mechanisms is the oxidation of cellular membrane by the process of lipid peroxidation. ROS like OH, HO$_2^-$, and OONO$^-$ can attack a methylene group binding a proton and leaving a carbon radical in the lipid. This initial carbon radical can then attack other methylene groups in lipid molecules, leading to a chain reaction, which can alter the integrity of lipid molecules in cell membrane [49]. ROS can also result in DNA strand breaks. The Fenton reaction, which includes iron or copper ions, can help to generate highly toxic OH$^-$ and then alter the transcriptional and translational process of DNA [50]. In addition, ROS can attack certain amino acids in proteins, some examples including reactions with tyrosine to produce 3-nitrotyrosin, or reactions with histidine, arginine, lysine and proline to generate a carbonyl group. These oxidation reactions can affect protein’s affinity to its receptor or enzymatic activity.

2.3.3. Redox homeostasis

Thus, the balance between ROS and antioxidant and the steady concentration of ROS is based on the balance between their production removal rates, which is currently referred to as redox hemostasis. Redox state, which refers to a concentration ratio of a redox pair, e.g. Glutathione
(GSSG/2GSH) or ascorbate (Asc+/AcsH'), describes the general condition of the cell. According to redox hemostasis, the redox state is maintained within a relatively narrow range under normal conditions. The redox homeostasis is attributed to the buffering capacity derived from GSH and thioredoxin (TRX). Glutathione acts as the major cellular redox buffer and is a representative indicator for the redox status of the cell. Under increased oxidative condition, GSSG concentration also increases. Thus increased GSSG concentration can interfere with the function of many critical proteins through the formation of disulfides from thiol groups. Examples of such proteins include receptors, protein kinases and some transcription factors. Besides GSH and TRX, many kinds of low molecular weight antioxidants can be significantly involved in ROS scavenging activities. This mechanism is mainly related to free amino acids, peptides, and proteins. It was found that oxidized proteins are much more vulnerable to proteolytic degradation [51].

### 2.3.4. Signaling of ROS

Recent studies showed that besides causing physical damage to biomolecules ROS also have a role as regulators of metabolic processes [45]. Signal transduction is a process enabling information to be transmitted from the outside of a cell to various functional elements inside the cell. Signal transduction is triggered by extracellular signals, such as hormones, growth factors, cytokines and neurotransmitters. Signals are designed to influence transcription machinery that is responsible for expression of a certain gene, and signals are normally transmitted to the cell
nucleus by a class of proteins called transcription factors. ROS were found to play a major physiological role in several aspects of intracellular signaling and regulation.

Growth factors, cytokines, or other ligands can induce ROS generation in non-phagocytic cells through the specific binding to membrane receptors. This signal transduction can often be enhanced by the ROS production or by a pro-oxidative shift of the intracellular thiol/disulfide redox state, which generates a kind of positive feedback. More specifically, it was found that nerve growth factor (NGF), epidermal growth factor (EGF), and Platelet Derivation Growth Factor (PDGF) can induce intra-cellular ROS through the signaling protein Rac1, which is a specific type of G-protein [52]. Also, growth factors’ functions are inhibited by hydrogen peroxide removal. There is a strong possibility that the redox dependency of the signal transduction process may facilitate synergistic interactions between different types of membrane receptors. For example, angiotensin II type I receptor, which is a G-protein-coupled receptor, was found to induce responses by ROS that are normally activated by tyrosine kinase-linked receptor.

Protein tyrosine phosphatases (PTP) counteract the effect of protein tyrosine kinases (PTK), which can dephosphorylate all tyrosine residues immediately after ligand-induced autophosphorylation and reset PTK receptor back to the normal situation. However, this phosphatase is inhibited by high levels of ROS by cysteine oxidation in cell, and thus ROS slows down the dephosphorylation and directly triggers PTK [53]. Then, increased level of tyrosine
phosphorylation co-regulates PTKs along with its ligands. For example, insulin receptor has higher sensitivity to insulin only with certain concentration of ROS. The activity of several protein tyrosine kinases such as LCK, Fyn, Syk, and ZAP70 was found to be regulated by ROS [31].

Similar to PTK, serine/threonine kinases can also be regulated by ROS concentration. Among serine/threonine kinases, PKC is significant to transcription and cell cycle control. This PKC was proven to be activated by hydrogen peroxide via cysteine-region domain. Akt, a serine/threonine kinase, is involved in PI3K and can be activated by phosphorylation. This mechanism has the effect on inhibition of an apoptosis pathway. Recently some studies proved that ROS participate in activation of this mechanism [54].

Mitogen-activated protein kinases (MAPK) signaling cascades are regulated by phosphorylation and dephosphorylation on serine/threonine residues and are activated by the either receptor tyrosine kinases, protein tyrosine kinases, receptors of cytokines or heterotrimeric G protein-coupled receptors. Different species of MAPK, including JNK and p38 are strongly activated by ROS or by a mild oxidative shift in the intracellular thiol/disulfide redox state. Two other enzymes, signal-regulated kinase 1 (ERK) and ERK-2 were found to be activated in vascular smooth muscle cells by superoxide [55]. Furthermore, studies on the up-regulation of MAPKs by H$_2$O$_2$ have demonstrated that activation is both type and stimulus specific. For example, endogenous H$_2$O$_2$ from respiratory burst can induce ERK but not p38 kinase activity, while
exogenous H$_2$O$_2$ activates p38 kinase but not ERK in rat alveolar macrophages [46].

The most significant effect of ROS on signaling pathway is thought to be on the MAPK pathway since it is involved in expression of the genes that control the immune system, proliferation and apoptosis.

The nuclear transcription factor NF-$\kappa$B is involved in inflammatory responses and AP-1 is important for cell growth and differentiation. p53 is a gene whose disruption is associated with cancers. Jun, Fos are the transcription factor of AP-1. The mRNA of Fos and Jun, (c-Fos and c-Jun) are found to be induced by relatively small amounts of hydrogen peroxide, superoxide, NO, and ROS. Cells treated with hydrogen peroxide can cause the expression of c-Jun and c-Fos and simultaneously increase the AP-1 transcription factor activity. The reason may be the fact that this oxidative activation of AP-1 is based on the activation of JNK, a kind of MAPK. On the other hand, NF-$\kappa$B is involved in so many various responses, including inflammatory reactions, growth control, and apoptosis [6]. The activity of NF-$\kappa$B can be inhibited by antioxidants such as cysteine, and can be activated by low concentration of hydrogen peroxide. That is because that ROS can mediate the enhancement of $\text{I}\kappa\text{B}$ (inhibitor) degradation via oxidant modification. On the other hand, ROS can influence the up-stream signal cascade.

Taking all above into consideration, ROS can interlace in the complex intracellular signal transduction network. Instead of being ligand or inducing factors which can activate one specific
molecule, ROS participate in many signaling pathways, either increasing or decreasing the effect of some pathways. Redox state is similar to other biological indexes, pH and temperature, which can be regulated by the system and can also regulate the overall cell performance.

2.3.5. ROS generated from exogenous free radicals

Tobacco smoke consists of two major fractions: solid (also referred to as tar) and gas phase. These two fractions can be separated from each other using a standard glass fiber Cambridge filter. Studies since 1980s have illustrated that gas phase and tar contain two very different populations of free radicals[56]. Free radicals from tar are exceptionally stable that can be observed directly by electron paramagnetic resonance (ESR) spectroscopy. Conversely, gas phase radicals are both less stable and short-lived, and therefore cannot be observed directly by ESR [57].

Early studies hypothesized that all related tarry materials contain a single type of radical consisting of an odd electron delocalized over a large aromatic quinone-like framework. Nowadays, it has been proven that, out of the tarry polymeric materials in tobacco, the principle radicals which give the major peak on the 2nd derivation of the ESR spectrum (g value from 2.0035 to 2.0038), consists of quinone (Q), semiquinone (QH), and hydroquinone (QH₂) [56]. Studies reported that the source of these quinone, semiquinone, and hydroquinone radicals is the condensation of catechol and 1,4-naphthoquinone, rather than polynuclear aromatic hydrocarbons (PAH) such as pyrene or anthracene [58].
According to the widely accepted hypothesis, superoxide and hydrogen peroxide result from the reduction of oxygen by the hydroquinones and semiquinone radicals present in tar. However, different groups used different equations to describe this mechanism.

\[ Q + QH_2 \rightarrow 2H^+ + 2Q^- \]

\[ Q^- + O_2 \rightarrow Q + O_2^- \]

Or

\[ QH_2 + O_2 \rightarrow QH^- + O_2^- + H^+ \]

\[ QH^- + O_2 \rightarrow Q + O_2^- + H^+ \]

Or

\[ Q + QH_2 \rightarrow 2QH^- + O_2^- + H^+ \]

\[ QH^- + O_2 \rightarrow Q + O_2^- + H^+ \]

In summary, no matter which equations are used, researchers agree on the fact that, Q/QH−/QH₂ system can generate semiquinone and/or quinone radicals spontaneously, which can reduce oxygen to form superoxide \( O_2^- \). Later this high energy radical can reduce \( H^+ \) ions to form hydrogen peroxide [58].

\[ 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \]
H₂O₂ can then be reduced to the hydroxyl radical (HO⁻) by metals such as iron (Fenton reaction).

Furthermore, studies presume that the lack of fine structure in the ESR spectrum may attribute to the rapid hydrogen atom transfer between the different quinone oxidation states. A polymer of modest molecular weight present in tar would contain various quinone (Q), semiquinone (QH), and hydroquinone (QH₂) species. And these species can readily interconvert via hydrogen atom exchange in such a complex Q/QH/QH₂ system (Figure 3) [59].

Figure 3. Schematic representation of the Q/QH/QH₂ equilibria in Q/QH₂ polymers in tar.
Further investigation led to the hypothesis that the tar radical system is an equilibrium mixture of semiquinones, hydroquinones, and quinones. It is suggested that this free radical complex causes redox cycling that generates superoxide anion from molecular oxygen and leads to the continuous formation of hydrogen peroxide and hydroxyl radical.

On the other hand, tar is a highly sticky substance and it tends to gel along the nasal and tracheal passage until the tar reaches alveoli in the lung. Tar deposits in the respiratory tract and initiates a long-term generation of free radicals, which promote the inflammatory response. The adverse effect of tar can be accumulated along over the years of smoking, owing to the long retention time of the tar in the respiratory system [58].

2.4. Novel pharmacotherapy of COPD

As mentioned above, the traditional anti-inflammatory drugs, corticosteroids, have been proven effective in asthma but found ineffective in COPD. The existing bronchodilator therapy is directed to relief of symptoms, which has limited ability to cure the inflammatory reaction in the lung. Thus, there is currently no effective treatment for COPD. Therefore, more effort is required to develop new more efficient drugs for COPD.
2.4.1. Mediator antagonists

The network of inflammatory mediators, including cytokines, growth factors, ROS, and lipid mediators, induces chronic neutrophil inflammatory in COPD. If one were able to cut off some of the links between the mediators and inflammatory cells by using antagonists or inhibitors, COPD could be attenuated. These potential agents are summarized in Table 1 as other anti-inflammatory drugs.

**Lipid antagonists**

$\text{LTB}_4$, as mentioned above is a leukotriene, which is derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A2, 5-lipoxygenase (5-LO) and leukotriene A$_4$ (LTA$_4$) hydrolase. Macrophages and neutrophils are the main source of $\text{LTB}_4$, and are the subjects of its recruitment function. $\text{LTB}_4$ receptor antagonists such as LY29311, SC-53228, CP-105, 696, and so on, are found to inhibit the neutrophil chemotactic activity [60]. They are mainly the antagonists of BLT1 receptor. Most of them showed significant anti-inflammatory effect in a variety of diseases [61]. However, clinical studies on COPD have not been that positive. A study by Gronke et al. attempted to evaluate the effect of LTB019 (one $\text{LTB}_4$ receptor antagonists) on inflammatory markers of COPD [62]. A 4 week double-blind, randomized, crossover trial of LTB019 in 24 patients found that there were no significant differences between LTB019 group and the placebo. The outcomes were the percentage of sputum neutrophils, total cell count, and the
level of MPO, IL-8, and TNF-α. In addition, the FEV₁ and FVC are also considered. Thus there is still no evidence that LTB₄ inhibition could be an effective treatment for COPD [63].

5-LO is a crucial enzyme in the production of leukotriene, thus its inhibitor has potential to reduce the inflammatory. Some of 5-LO inhibitors act by interfering with the conformation of the active sites while others act as antagonists to block the substrate binding site of the enzyme. However, it has been difficult to develop a potent safe 5-LO inhibitor [64]. Thus, in the foreseeable future, the application of lipid antagonist will still be limited.

**Chemokine antagonist**

The trafficking of all inflammatory cells is orchestrated by multiple chemokines. Thus, blockage of chemokine receptors with selective antagonists might be an effective anti-inflammatory strategy. Their potential is further confirmed by studies in animal model of knocked out CXC receptor genes.

IL-8 is the most important chemokine in COPD inflammatory. As illustrated above it can activate neutrophils via CXC receptors. Thus, one possible therapeutic approach could be the use of an IL-8 antibody, and several studies illustrated that anti-interleukin-8 monoclonal antibody derived from human showed inhibition of neutrophils inflammatory in animals and reduced the chemotactic response of neutrophils. However, it is known that IL-8 activates neutrophils via low-affinity G-protein-coupled receptor (CXCR1) and plays a chemotactic role via high affinity
G-protein-coupled receptor (CXCR1). Moreover, other chemokine also can activate CXCR2, and CXCR2 is also presented on monocytes. Thus, CXCR2 antagonists are more useful than IL-8 antibody [65]. Amino-terminal truncation of IL-8 and amino-acid modification are first produced as CXCR antagonists. Several small-molecule inhibitors have been developed, including CXCR2 antagonists (e.g. SB332235), CCR2 antagonists (e.g. RS504393), CXCR3 antagonists (e.g. NBI74330), and CCR5 antagonists (e.g. TAK220). These antagonists effect diverse therapeutic effect on inflammatory [66]. The first small-molecule, non-peptide antagonist of CXCR2 is SB225002, and it has selective affinity to CXCR2 [67]. Later many in vivo studies showed efficacy of CXCR2 antagonists in IL-8-induced neutropenia in rabbits. Improved in vivo performance was achieved by a CXCR2 selective antagonist of the same chemical series [68]. Later, more CXCR2 receptor antagonists were developed, such as pyrazines/quinoxalines and growth-regulated oncogene [69, 70] Although several papers highlight the dominating role of CXCR2 in COPD and mention the potential of CXCR2 antagonists in the therapeutic of COPD, the performance of CXCR2 antagonists in lung inflammatory was not established.

Many studies focused on another receptor antagonists, AMD3100, an antagonist of CXCR4. Its effect in the reduction of allergic inflammatory in the lung is proven, and the mechanism of action is decreasing of chemotaxis of lymphocytes. In addition, some antagonists for receptors to CC chemokines may be of use since CC chemokines also play roles in recruitment of inflammatory
cells in COPD [71]. Similarly, limited studies are focused on anti-inflammatory ability in COPD or even other lung inflammatoryys like bronchiolitis and asthma.

Even current studies hold considerable promise for the treatment of COPD by antagonists, but additional systematic research is still necessary before the their application [72].

**Cytokine inhibitor**

TNF-α is an important cytokine in inflammatory, and thus TNF-α inhibitors are considered as potential anti-inflammatory drugs. Infliximab is one FDA approved TNF-inhibitor. Infliximab was initially produced for the treatment of Crohn’s disease. It is a novel chimeric monoclonal antibody, anti TNF-α, which has both human and mouse fragments. This chimeric antibody has later shown a therapeutic effect in patients with rheumatoid arthritis, ankylosing spondylitis, Crohn’s disease and psoriasis [73].

In patients with COPD, the level of TNF-α in the sputum and blood are increased, and the genetic polymorphisms in the promoter regions of the TNF-α gene has been associated with chronic bronchitis [74]. In addition, the increased on TNF-α level is found to be related to the weight loss of the patient. Thus, blocking of TNF-α might have a beneficial response. Furthermore, the study with mice lacking TNF-α showed less emphysema compared to the wild type after exposure to smoke for 6 months [75-78].
However, studies showed no therapeutic benefit in the evaluation of the efficacy of infliximab in human subjects with moderate to severe COPD, not supporting the use of infliximab for COPD. The impact of infliximab on malignancy risk in patients with COPD needs to be further elucidated [79]. All in all no confirmed answer to the ineffectiveness of infliximab to COPD [80].

**Antiproteases**

Proteases, as mentioned above, play an important role in COPD, especially in emphysema. The imbalance between proteases and antiproteases, leads to the destruction of the lung tissue in the chronic phase of COPD. Thus, great effort has been directed to restore this balance through the use of low molecule weight human elastase inhibitors. As illustrated above, neutrophil elastase (NE) and Matric metalloproteinases (MMP, especially MMP-9) are two dominate proteases in COPD.

For the inhibition of NE endogenous inhibitors were initially studied. One paper summarized that there are at least three endogenous human neutrophil elastase inhibitors, including α1-PI, secretory leukocyte protease inhibitor (SLPI) and elafin (also called elastase specific inhibitor (ESI) or skin-derived antileukoprotease (SKALP) [81]. SLPI shows its protective effect against protease mainly in the upper respiratory tract while α1-PI probably works in the lower pulmonary field. Inhalation of SLPI with α1-PI were found beneficial in the treatment of inflammatory lung disorder, including emphysema, chronic bronchitis, and cystic
Later, synthetic neutrophil elastase inhibitors were developed, which are divided into two categories: acyl-enzyme inhibitors and transition-state inhibitors. These molecules are considered to be the new drugs for COPD. NE attacks the reactive carbonyl carbon of the acyl-enzyme inhibitors, forming a tetrahedral intermediate. Later this intermediate collapses and forms a relatively stable acyl-enzyme complex, thus preventing further hydrolytic process catalyzed by NE. Many acyl-enzyme inhibitors are developed including: ONO-5046, MR-889, L-694,458, CE-1037 and GW-311616 [82]. Most of them have been discussed as a potential treatment of COPD. Some of them have been clinically tested for COPD. A 4 week double-blind randomized and placebo-controlled clinical study was performed with MR-889. The safety of administration was proven, but the therapeutic effect on COPD was not obvious [83]. Another group of synthetic NE, transition-state inhibitors, is attacked by NE on the ketone carbonyl and forms a stable tetrahedral intermediate. Also many transition-state inhibitors are in development, but limited data of their effect of COPD is available.

Another important group of proteases in COPD is MMP. Many MMP inhibitors have been developed including hydroxamate-based MMPIs, such as BB-1101, ADAM17, and CGS27023A, and non-hydroxamated-based MMPIs such as BAY12-95666. One recent study analyzed the effect of one MMP-9/12 inhibitor, AZD1236, on COPD [84]. AZD1236 is a potent, orally bioavailable
inhibitor of MMP-9 and MMP-12, which showed high selectivity for these MMPs in vitro. From this randomized, controlled, double-blind, multinational study, the trend of a reduction of desmosine in both blood and urinary was shown, suggesting the possibility of a reduction in elastin, but no significant therapeutic effect was demonstrated from the majority of biomarkers [85, 86].

Despite of all these results obtained from these studies, various problems that remained to be solved in the clinical use of proteases inhibitor

**NF-κB inhibitors**

According to the previous discussion, NF-κB plays an important role in the expression of many pro-inflammatory mediators, for example IL-8, TNF, and metalloproteinases, so the inhibition of NF-κB seems useful to COPD therapy. Many ideas have been applied on NF-κB inhibition, including gene transfer, inhibition of IκB kinase, and inhibition of NF-κB inducing kinases. However, inhibition of NF-κB is always expected to have a side effect of the compromising of the immune system [87] [88].

NF-κB induced gene expression can be triggered by a variety of physical and chemical stimuli including cytokines, viral irritates. ROS, etc. and is controlled by a complex series of proteins and enzymes. In resting cells, most NF-κB is bound to a protein inhibitor IκB which can keep NF-κB in the cytoplasm. Triggered by the stimuli, the IκB is phosphorylated and ubiquinated leading to a release of NF-κB. Free NF-κB can translocate to the nuclei and bind to
specific DNA sequence, inducing gene transcription. The critical phosphorylation of the IκB is performed by IκB kinase (IKK) complex. IKK is known to consist of two catalytic subunits (IKK-1 and IKK-2) and a regulatory subunit IKKγ. IKK-2 is found to be 20-fold as active as IKK-1 in the phosphorylation of IκB, and IKK-2 also can directly phosphorylate IκB. For this reason, there has recently been studies for small molecular weight inhibitor of IKK-2 to inhibit the inflammatory reaction from NF-κB [89] [90].

Several low molecular weight IKK-2 inhibitors have been developed. Thalidomide and its analogs have shown potential anticancer, anti-inflammatory, anti-angiogenic, and immunosuppressive effects. Other small molecule inhibitors, including 15d-PGJ2 and PGA1, inhibit IκB degradation via forming a direct covalent bond to IKK-2 via cysteine 179 within its activation loop. Later more selective inhibitors were developed, for example SPC-839, PS-1145, and BMS-345541 [90].

In addition, other groups of inhibitors were developed. e.g. inhibitor of ubiquitination that also block IκB degradation. Other inhibitors were found to prevent binding of NF-κB to DNA by modifying NF-κB active site or occupying the binding site for NF-κB on DNA [91].

Among all these NF-κB inhibitors, IKK-2 inhibitors are the dominant and most developed group. Some animal experiments were designed to study potential effect of these inhibitors in COPD. In one study, a highly selective IKK-2 inhibitor, PHA-408 was orally administrated to
rats exposed to LPS or cigarette smoke. Reduced inflammatory response rate was demonstrated from the inhibitor, which is the first evidence that administration of IKK-2 inhibitor exerts anti-inflammatory effect in vivo in rodent lung against cigarette smoking [92].

2.4.2. P38 MAP kinase inhibitors & Phosphoinositide 3-kinase inhibitors

When the inflammatory mediators activate the receptors on the cell membrane, the signal can pass down to the gene transcription level by various pathways. The mitogen-activated protein kinases (MAPK) plays a key role in these pathways. Thus, inhibitors of p38 MAPK have a broad spectrum anti-inflammatory effect. Moreover, the phosphoinositide-3 kinase inhibitor could also be a potent drug, since the knock-out of this gene is found to inhibit neutrophilic inflammatory. The inhibitor targeting to proteases is also promising in COPD therapy. Studies just claimed the P38 MAP kinase inhibitor as potential COPD therapies, and limited studies focused on its application in COPD or even on its anti-inflammatory efficacy [93] [94].

2.4.3. Non-enzymatic antioxidant

Exogenous ROS is known to be the initial main causal factor for tobacco induced COPD, and endogenous ROS amplifies the inflammatory in COPD. Therefore the anti-oxidants can be considered as potential therapeutic agents. Compared to all antagonists and inhibitors discussed above, antioxidants, which may reduce the causal factor in COPD inflammatory, have recently attracted much attention in COPD treatment.
In a normal lung, GSHs are known as the buffer to defend the oxidative stress, and therefore raising the GSH level by drug administration was considered by several groups. GSH can consume ROS and convert into GSSG. However, direct administration, either via aerosolization or oral administration, was found inefficient due to the short half-life and limited cell uptake of GSH [95]. An alternative approach of using a GSH precursor or another cysteine supplier was therefore considered. Acetylcysteine also known as N-acetylcysteine or N-acetyl-L-cysteine (NAC) is one example of a cysteine supplier. Some benefits were found after NAC oral administration, but the effects were marginal, and the dosage for potential clinical benefit in COPD still remained to be determined [96]. An alternative to NAC is N-acystelyn which can increase GSH more effectively and is neutral in solution. In addition, other GSH donors have been explored as antioxidants. These include erdosteine, fudosteine, and carbocysteine, which have the ability of reduction exacerbations rates and improvement of life quality in long term [97]. However, recent studies showed that, these antioxidants either were proven no beneficial in COPD patients, or still required further evidence to illustrate their therapies[98].

2.4.4. Enzymatic antioxidant/ SOD

The redox imbalance in COPD mentioned above is not only caused by over-production of ROS or too much exogenous ROS, but also by the limited level of antioxidant enzymes and antioxidant small molecules in the affected respiratory tissue. Thus, a strategy of exogenous
delivery of antioxidant enzymes to neutralize ROS in lung tissue could be a viable approach. The most important ROS-detoxifying enzyme present in cells is superoxide dismutase (SOD). It catalyzes the breakdown of superoxide radicals into less toxic oxygen and hydrogen peroxide, the latter being further reduced by glutathione peroxide or catalase [99]. The metal cofactor of SOD plays a role as an electron acceptor. Three major families of SOD are defined based on their metal cofactor; the classes including copper/zinc SODs, iron/manganese SODs, or nickel SODs.

\[
M^{(n+1)}\text{-SOD} + O_2^- \rightarrow M^{n}\text{-SOD} + O_2
\]

\[
M^{n}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)}\text{-SOD} + H_2O_2.
\]

Many studies on mice and humans have proven the anti-inflammatory effect of SOD on many chronic inflammatory diseases, including myocardial ischemia, reperfusion injury, arthritis, secondary spinal injury, Parkinson’s disease, and Alzheimer[100-102].

Some researchers focused on the anti-inflammatory effect of SOD in COPD. Studies have proven that airway epithelium can be triggered by second-hand smoke exposure to release IL-8 in vivo [103]. Mulligan RM et al, from the detection of IL-8, demonstrated that CSE (cigarette smoke extract) is able to raise the inflammatory reaction on both upper and lower airway epithelium which mimetics the situation in COPD. This pro-inflammatory reaction can be attenuated by SOD in vitro [104]. According to this result, delivery of SOD to respiratory system could reduce the inflammatory induced by tobacco usage in COPD.
The safety and the anti-inflammatory effect of SOD have been proven by a double blind, placebo-controlled clinical trial of PC-SOD, phosphatidylcholine SOD [105]. Tanka et al, found that not only intravenous administration but also inhalation of PC-SOD ameliorated pulmonary inflammatory, emphysema, and dysfunction, through suppression of cell death, decrease of proteases, reduction of expression of proinflammatory cytokines and chemokine, and increase the level of antiprotease in an animal model study. Thus, this modified SOD molecule was therapeutically beneficial for COPD [106].

2.5. Inhalation drug delivery

An effective method to deliver drugs to the specific site of tissues or organs is always challenging for pharmaceutics, especially for protein/peptides drugs, since their activity is closely related to the conformation, and is very vulnerable to the gastrointestinal enzymes and the phagocytic system in the circulation [107]. This leads to several new delivery approaches, and pulmonary delivery is one of them. Basically, there are two kinds of pulmonary delivery, nasal and oral inhalation drug delivery [108, 109].

The most obvious advantage of pulmonary delivery in COPD is its noninvasive nature, which is especially important for some fragile drugs including proteins and peptides. In addition, pulmonary delivery is a local delivery for respiratory diseases, which can have on site effect
immediately after administration. This technique has been used to manage asthma and COPD for more than 50 years. The earliest therapeutic agents, β2-agonists (LABAs) and inhaled corticosteroids (ICSs) for asthma patients were initially delivered directly to the lungs. The inhaled route offers a more rapid action compared to any systemic administration including injection. The lung is an ideal site for inhalation delivery because of its high solute permeability, large absorption area, and limited proteolytic activity. The solute permeability is extremely low in the lung compared to other organs [110]. The surface area of the lung ranges from 75 to 140 m² mainly from 500 million alveoli, while 2300 km respiratory tract has surface area of 0.25 m². Due to local delivery, the required therapeutic dosage of an inhaled drug can be decreased, thereby increasing the safety of administration [111].

Because of the obvious advantages of the inhalation delivery route, it has also been investigated by many research groups for systemic drug delivery [112-115]. This route offers many advantages over the oral or injection administration for systemic drugs as the mucosa possesses a well-developed vascular and lymphatic drainage system and the high permeability of the lung and large absorption area also favor absorption of the systemic drug. Lastly, but most importantly, inhalation drug delivery can circumvent first-pass metabolism in the liver and the pre-systemic elimination in the GI tract [116].

Nasal delivery is a special case of inhalation drug delivery. The same as traditional oral
inhalation, nasal delivery is originally applied for treatment of local diseases such as nasal allergy, nasal congestion and nasal infection. Later, its systemic application has been exploited especially for proteins and peptides, which are not easily administered by other routes other than by injection [117] [118]. There are also two special applications where nasal delivery is especially important, vaccines and nose to brain delivery. Nasal delivery of vaccines is promising especially for diseases whose pathogenic microorganisms enter across the respiratory surface. The nasal mucosa is the first site of contact with inhaled antigens and is also linked to nasal associated lymphoid tissue (NALT), which can create both mucosal and systemic immune responses. On the other hand, the possibility of nose to brain drug delivery offers one encouraging method to bypass the blood-brain barrier (BBB) and reach the central nervous system directly from the nasal cavity. Many researchers believe that a possible mechanism involves uptake by olfactory region rather than direct diffusion to brain tissue or to cerebro-spinal fluid. Delivery efficiency is mainly dependent on the molecule size and lipophilicity. Still many efforts are needed to enhance the fraction of the drug that reaches the brain [117].

2.5.1. Size related particles deposition in lung

Though pulmonary delivery could be an ideal treatment for respiratory diseases, the drug deposition site still needs to be addressed. For example, it has been proven that, although the β₂ agonist receptor is mainly located at the alveolar wall, the smooth muscle located along the airway
and terminal bronchioles is the major targeting site of these bronchodilators. In contrast, other
drugs are mainly targeting to either conducting airways or to the alveolar region. Since COPD
includes several different pathological mechanisms and the inflammatory occurs mainly at the
small airways and alveolar region, these would be ideal sites for anti-inflammatory drugs.

Despite the recent development of aerosol techniques, there are still many complex barriers,
which have to be circumvented if drugs need to reach the deep lung. The first one is the airway
geometry of the lung. The lung resembles an inverted tree, where the trachea or trunk subdivides
into two main bronchi and later consecutively branches into much more narrow and shorter
bronchioles. Specifically, the trachea undergoes 23 bifurcations before it reaches the alveolar sacs.
The first 16 generations are divided into the conducting region where the air is supposed to be
filtered and humidified. The rest is the respiratory region, which is mainly responsible for gas
exchange, and includes bronchioles, alveolar ducts, and the sacs. Therefore, the drug is likely to be
filtered before it goes deep enough into the lung via absorbing on the airways. Thus, many studies
focused on the fate of inhaled drug particles [119].

The site of deposition directly affects the efficacy of the inhaled drug. It is influenced
primarily by three factors: (a) particle/droplet size, coupled with density, surface properties, and the
shape; (b) anatomy of the upper and lower airways and the alveolar structure; (c) ventilator
parameters of the breathing pattern. The deposition of the particles along the airway is ruled by
three principally different mechanisms: inertial impaction, gravitational sedimentation, and Brownian diffusion. The size and geometry of the particle affect the aerodynamic diameter, which can be conceptualized by considering a spherical particle settling under gravity through air, and are quite different from the geometric diameter. It is defined by the equation:

\[ d_a = d_g \sqrt{\frac{\rho}{\rho_a}} \]

Where \( \rho \) is the mass density of the particle, \( \rho_a \) is the unit density (1000 kg/m\(^3\)) and \( d_g \) is the geometric diameter. Practically, a mass median aerodynamic diameter (MMAD) is applied to indicate the inhaled particle size [119]. It is difficult to predict the actual site of deposition, since anatomy differs among people, but from a general model and in vivo experiments, particles with a MMAD of 5-10 \( \mu \)m are mainly deposited, or blocked at the large conducting airways and oropharyngeal region (also called upper airway), by inertial impact. Particles with 1-5 \( \mu \)m in diameter are deposited on small airways and can partially reach alveoli, and particles with < 3 \( \mu \)m in diameter have a chance to reach the respiratory bronchiole with around 50% of them depositing in the alveoli. These particles’ deposition is mainly dominated by gravimetric sedimentation. Any particles smaller than 1 \( \mu \)m, can remain suspended in the air, which can reach the lung easily and deposit in the lung by random diffusion. However, large fraction of too small particles (< 0.1 \( \mu \)m) can be lost due to exhalation. Moreover, particles < 0.01 \( \mu \)m are blocked at the tracheal-bronchial region due to too high diffusion coefficients (Figure 4) [120]. As mentioned above, since different
drugs have distinct targeting site, they are supposed to be formulated into different particle sizes. Thus, inhalers generate micron size particles for the tracheal-bronchial tree (3-10 µm) or in the alveolar region (1-3 µm) for systemic drug. Since anti-inflammatory targeting site is in the deep lung, small sized particles are required to be formulated [121].

Figure 4. Relationship between particle size and lung deposition: the trachea-bronchial (T-bronchial) region consists of the windpipe and large airways; and the pulmonary region consists of the small bronchi and the alveolar sacs.

2.5.2. Mucus clearance in the airway region

Once particles are deposited onto the epithelial lining of the lung, they first contact the mucous layer within the airway or the surfactant-coated fluid layer within the alveolar region. The mucus clearance system provides one of the most significant defense mechanisms against inhaled
materials by removing and/or neutralizing alien toxic materials [122]. However, this also acts as the strongest hindrance that limits the pharmaceutical efficiency of respiratory drug delivery. Mucus (about 5µm in depth) is a viscoelastic gel layer that is exposed to the external environment and is mainly composed of a three-dimensional network of cross-linked glycoprotein mucin secreted by goblet cells and submucosal glands (Figure 5) [123-125]. Mucin monomers, each about 0.3-0.5 MDa are highly cross-linked or entangled, which gives the mucus its viscoelastic properties. There is significant polydispersity in mucin’s molecular weight, and the data from different studies show a wide range from 0.2 to 40 MDa. Mucins are coated with the diverse array of proteoglycans [126] and thus they are negatively charged. Several systems are available to classify mucin fibers, but generally they can be divided into two families: cell-associated mucins ranging between 100-500nm in length, which contains a transmembrane domain, and secreted mucins that are up to several microns long [126-128]. In addition to mucins, mucus gels are loaded with cells, bacteria, lipids, salts, proteins, macromolecules, and cell debris, with a shear dependent bulk viscosity that is typically $10^3$-$10^4$ times higher than that of water at low shear rates [129].

The mucus layer acts as the barrier to drug delivery, both local and systemic, via several mechanisms, and these mechanisms supplement each other. First, the mucociliary clearance rapidly removes the drug formulation from the lung to the gastrointestinal tract (Figure 5). Bacteria, irritants and particles are stuck to the viscous mucus, and then are transported either downward or
upward to the pharynx and later swallowed into the esophagus. The transportation is closely related
to the beating of the cilia present on the respiratory epithelial cells. With a beat of ~1000 strokes per
minute, the cilia pushes the mucus at a speed of ~5mm per min [130], making the half-life of a
polar drug such as protein very short (15-20 min). Secondly, the cross-linked mucin network
hinders the diffusion of a drug by steric obstruction, or by surface binding. This mechanism
constrains a substantial fraction of a polar drug in the upper layer of mucus, which has shorter
turnover time, compared to a lower layer of mucus. It also compromises the drug therapeutic
efficiency if the drug was designed to act inside the epithelial cells [129]. Finally, the drug trapped
in mucus is vulnerable to enzymatic degradation, especially if it is a peptide or an enzyme.

Thus, mucociliary clearance effectively eliminates particles that are unable to penetrate the
mucus network, which has pore diameters ranging from 20 to 800nm.
Figure 5. Components of the mucociliary system: the cilia (hair type projections of the epithelia cells), the periciliary fluid layer and the mucus. The mucus is secreted from goblet cells and the submucosal glands. Mucociliary clearance is the primary mechanism of clearance of mucus [131].

Thus, in order to efficiently deliver a protein drug such as SOD to the site of action and to achieve optimum therapeutic performance, special drug delivery techniques must be utilized. Unlike agonists and corticosteroids, which are hydrophobic, proteins are polar drugs and as such are more likely to be hindered by the mucus layer.

Many strategies have been explored in an effort to overcome these barriers by the
modification of the peptide structure, the inhibition of the ciliary beat frequency, employment of permeation or absorption enhancers, and the utilization of mucoadhesive polymers. Among these strategies, improving mucoadhesion of particles in order to improve their retention at mucosal surfaces is often utilized. This is due to the fact that mucus renewal is much slower than the transit time of the luminal content. A variety of mucoadhesive drug delivery systems have been engineered, driven by various interaction forces between mucus and drug particles, including hydrogen bonding, van der Waals interactions, polymer chain interpenetration, hydrophobic forces, and electrostatic interactions [132]. Nano-carriers have been considered as one of the tools to improve respiratory drug delivery. Nano-size particles are favored because of their ability to be trapped in mucin networks. As one example of a system that utilizes electrostatic interactions, chitosan nanoparticles were exploited to improve mucoadhesion. Chitosan is cationic polysaccharide composed of d-glucosamine and N-acetyl-d-glucosamine units. Mucus, as described previously, is negatively charged, and can have ionic interaction with the positive amino groups of chitosan. Chitosan can also enhance the paracellular absorption of polar drugs by reversible opening of the tight junctions between the epithelial cells [133]. Several studies have shown the application of chitosan as a nano-carrier that by provides a longer contact time for drug transport across the mucus layer and endothelium membrane, before the formulation is cleared by mucociliary clearance mechanism [134].
However, other studies illustrated several fundamental limitations of this approach. The most significant one is the toxicity of cationic nano-carriers. Mucoadhesive nano-carriers can substantially alter the microstructure of mucus, and this disrupted mucus barriers cause greater exposure to foreign materials, including pathogens and toxic materials.

Since the upper layer mucus (luminal mucus layer) is cleared more rapidly than the lower layer mucus (adherent mucus layer) many recent studies are planned to achieve longer residence time of particles by penetrating the luminal mucus layer, thereby reaching the adherent mucus layer, and hence having a better chance to be internalized by epithelial cells [129].

For mucus penetration, particles must be nano-sized to diffuse through the mucin network, and minimize hydrophobic entrapment by mucus. On the other hand, either anionic or cationic charged particles may exhibit immunogenicity. Thus, in order to circumvent the immunogenicity of delivery system, high hydrophilicity and neutral charge particles should be used. Poly (ethylene glycol) (PEG) modified nanoparticles were reported as the agents able to penetrate the luminal layer [135-137]. It has been argued that the improvement of drug delivery by PEG was most likely due to the improved mucus penetration properties, rather than the mucoadhesion mechanism, since the MW of PEG, around 2~10KDa, is too low to support adhesion via polymer interpenetration, and the hydrophobic core of nanoparticle is well shielded by the PEG chains. According to an in vitro study [65], the nanoparticles covered by dense and low MW PEG in fresh human undiluted
mucus can achieve very high transport rate, which is only 6-4 times lower than the same particles in water.

2.5.3. Clearance in alveolar region

Depositions on the alveolar region may be phagocytized and cleared by alveolar macrophages or absorbed into the pulmonary circulation. Alveolar macrophages (AM) are the primary defense system against inhaled microorganisms and toxic materials. The airside surface of each of the 500 million alveoli in the human lung is guarded and routinely monitored by 12-14 AMs. The deposited particles are either transported through alveolar thin epithelial cells into the systemic circulation, or phagocytized by AM and later cleared by ciliated airways or lymphatic system. According to the lung physiology, this process can take from weeks to months, which could be quite enough for drug delivery. In alveolar, for most protein drugs proteolytic degradation is not the main clearance mechanism. In addition, nanoparticles demonstrated ability to escape from AM on some scale and further prolong the retention time of drugs [81].

2.5.4. Advantage of nanoparticles in inhalation delivery

The main unique characteristic of nanomaterials is their size, which falls into the transitional zone between individual molecules and the bulk material. The high surface to volume ratio leads to more reactive groups on the surface. The use of nano-sized drug delivery vehicles (e.g. nanoparticles or micelles) have led to interesting innovations in pharmacology. A number of
nano-devices suitable for drug delivery made either of lipids or polymers are exploited for therapeutic purpose to carry the drug in the body in a controlled manner from the site of administration to the therapeutic target [138, 139]. In general, nano-carriers may (i) protect a drug from degradation, (ii) enhance drug absorption by facilitating diffusion through epithelium, (iii) modify pharmacokinetic and drug tissue distribution profile, and, (iv) improve intracellular penetration and distribution, (v) a relatively uniform distribution of drug dose, (vi) enhanced solubility of drugs, (vii) sustained release, (vii) decreased incidence of side effects, and (ix) improved compliance.

Specifically, in a respiratory drug delivery system, nano-sized particles can offer advantages in preventing deposition too early on the conductive tracts in respiratory system. In addition, specially coated nanoparticles can penetrate the gel layer of mucus and escaping from macrophages in respiratory system, prolonging the residual time. Many kinds of polymeric nanoparticles have been formulated for pulmonary delivery mainly via nebulizers.

2.5.5. Inhalation drug delivery devices

Drugs have to be specially formulated to direct their entry to the respiratory system rather than to the digestive system. This modification technique is called aerosolization. Modern aerosol techniques provide stable dispersions of either solid or liquid drug in a gas medium. The drug compound or the drug solution has to be broken up into small particles or droplets to form an
aerosol that can be inhaled with air. Energy is required to counter the attractive force (hydrogen bonds or van der Waals force) in solid drug or a solution and to form new surface of a smaller particle or droplet. After these aerosolized particles are inhaled, they will deposit along the airway of the lung by different mechanisms including gravitational sedimentation, inertial impaction and diffusion. Currently, there are 3 common aerosol devices, which are used for pharmaceutical application: Nebulizers, metered dose inhalers (MDI) and dry powder inhalers (DPI) [140, 141].

Nebulizers are among the traditional aerosol devices. One aerosol generator can formulate drug dispersion in air and vent it to a mouth piece or mask. Aerosol is taken by a patient by continuous normal tidal breathing. Based on different operating principles, nebulizers fall into two categories: jet nebulizers or pneumatic nebulizers. Jet nebulizer uses high-speed air flow through a nozzle that draws a liquid containing the drug and breaks the drug solution into droplets. In pneumatic nebulizers, high frequency acoustic wave is used as energy source to break the drug into droplets [108].

The most significant disadvantage of a nebulizer is inconvenience and lack of portability, which limits it to uses mostly in a clinical setting. Thus, other portable unit-dose inhalers were designed for household or everyday uses. One example is MDI, which contains pressurized and liquefied gas, called propellant. Drug is suspended as a colloid or dissolved as a solution in this propellant. When the propellant is released from the metered valve, it evaporates immediately,
leaving dry drug particles, which can be inhaled. On the other hand, DPI is a passive inhaler, which uses patient intake of air to entrain and disintegrate drug. The increased use of DPIs is owed to the environmental concerns about chlorofluorocarbons that are commonly used as propellants in MDIs. MDIs or DPIs is more difficult to use than nebulizers and requires appropriate technique. As a result, a significant fraction of the asthmatic and COPD patients are unable to use MDI or DPI effectively. Thus, training is necessary for the patients to have the correct technique. In contrast, though traditional nebulizers are not as convenient, they provide continuous delivery over several minutes rather than a single inhalation, and are much less dependent on the patient inhalation technique.

Traditionally, the nano-sized drug delivery vectors were administered via nebulizers, using aerosols with particle size in the range of 1-10 μm, each particle containing hundreds of liposomes/nanoparticles with the size in the range of 100nm.

However, currently other disadvantages have gathered attention from the nebulizer. Besides the inconvenience of the nebulizer device application, the stability of liposomes or other nano-sized vehicles is compromised in colloidal suspension. The energy introduced by nebulizer can induce the alternation of structure of nano-sized vehicles. Therefore, DPI was much preferred as the approach to deliver liposomes or even other kind of nano-sized vehicles into lung.
CHAPTER 3
SPECIFIC AIMS AND SIGNIFICANT

3.1. Overview

Glucocorticoids have been successfully utilized for the suppression of inflammatory in several chronic inflammatory diseases including asthma, rheumatoid arthritis, and inflammatory bowel disease. However, even a relatively high dosage of glucocorticoids results in a limited efficacy in COPD compared to their effect in asthma or other inflammatory diseases. It was found that the efficacy of glucocorticoids in COPD is compromised by the oxidative pressure in COPD patients, and this effect knew as glucocorticoid resistance. We hypothesize that antioxidants can restore glucocorticoid function by neutralizing the ROS. Thus, simultaneous administration of antioxidants and glucocorticoids could be an effective approach to the treatment of COPD. The long-term goal of this study is to achieve a significantly improved anti-inflammatory effect from antioxidants and glucocorticoids.

For the purpose of this study, dexamethasone (DEX) will be used as the model glucocorticoid drug because of its previous successful application in treatment of another inflammatory condition in the lung (asthma). Superoxide dismutase is a broadly used antioxidant that has been studied and shown efficacy in various inflammatory conditions [142]. In addition, low molecular weight SOD mimetics are a promising alternative to SOD because of their higher stability and better
intracellular uptake. Thus, these two molecules will be studied here as model antioxidants. Finally, administration of free drugs is not expected to be efficient due to mucociliary clearance, and therefore specially designed targeted nano-carriers will be utilized for simultaneous delivery of both DEX and SOD/SOD mimetic to ensure prolonged residence time and sustained release of drugs. Anti-mucin (anti-MUC1) antibody immobilized on the surface of the nano-carriers will be used as the anchoring agent. The specific aims of this study are outlined below.

3.2. Significance

Chronic Obstructive Pulmonary Disease (COPD) is the fourth leading cause of death with approximately 85% of these cases attributed to tobacco use [143]. Various toxins, especially free radicals from tobacco smoke, generate reactive oxygen species (ROS) in the respiratory system, causing a chronic abnormal inflammatory response that induces irreversible damage in the lung, specifically airways fibrosis and alveolar wall destruction. No effective therapy is currently available to prevent the exacerbation of COPD as current therapies only relieve the symptoms rather than offering a cure. Various experimental efforts to reduce the degree of inflammatory by using anti-inflammatory drugs have been proposed, but limited positive results from the clinical trials.
3.3. Innovation

- Although glucocorticoids have demonstrated encouraging therapeutic effects in the treatment of asthma and other inflammatory diseases, it is hampered by the oxidative stress resulting from COPD. Therefore, anti-oxidants administrated simultaneously with corticosteroids appear to be a promising approach in treatment of COPD. Co-delivery of these two distinct therapeutic molecules has been studied, the result showing a synergistic effect in cancer treatment; however, this approach has not been tested in treatment of COPD or other inflammatory diseases. This research addresses this need by proposing to co-deliver an antioxidant (SOD or SOD mimetic) and DEX using targeted nano-carriers as a delivery platform.

- Secondly, the proposed use of the anti-mucin1 antibody as a targeting ligand is a novel approach. Targeted delivery is well established for other drug delivery systems for intravenous administration, the targeting agents, for example an antibody, having the ability to direct the drugs to the specific tissue needing treatment. However, this mechanism has not been applied to inhalation administration. In the respiratory system, vigorous mucous cilia movement removes the drug from the lung in a short time. In this study, the proposed targeting antibody will selectively bind to mucin, which is the most abundant biomolecule on the surface of epithelial cells.
3.4. **Aim 1: Preparation of nano-carriers for the targeted delivery of antioxidant and anti-inflammatory drugs.**

This aim will focus on the preparation and characterization of the polymeric NPs and liposomes for loading the antioxidants and glucocorticoids. Several candidates of polymers and lipids will be investigated to form distinct nano-carriers, and various methods will be utilized to load drugs and targeting moieties. The subsequent analysis of the loading capacity and long-term stability will help to choose the appropriate type of nano-carrier and determine the optimal drug loading parameters. The information obtained in this aim is crucial for selecting the specific nano-carrier system for later study.

3.5. **Aim 2: The optimization of nano-carrier properties for maximum targeting efficacy**

The binding ability of an antibody-modified nano-carrier primarily depends on the number of antibody molecules attached per nano-carrier. Even though the targeting and binding effect of immune-modified nano-carriers has been addressed by many researchers, the antibody/nano-carrier ratio has seen limited attention. More quantitative and real-time analyses of
targeting are still needed for better understanding of the association and dissociation kinetics of immune-modified nano-carriers. It is possible that the addition of the first few antibody molecules may lead to a significant increase in the binding ability, while further addition may have moderate or no effect. This specific aim proposes to determine the optimal antibody/nano-carrier ratio for maximized binding.

3.6. Aim 3: In vitro evaluation of the protective efficacy of simultaneously delivered antioxidant and glucocorticoid drugs

Previous research has found that glucocorticoid drugs, which are effective in other inflammatory conditions, are blocked by oxidative stress in COPD, suggesting the potential to restore its anti-inflammatory function by delivering antioxidants and glucocorticoids simultaneously. Currently there is no cell culture or animal model of this glucocorticoid resistance. Therefore, one important goal of this study is to develop a cell culture model, which would help researchers to address glucocorticoid resistance induced by the oxidative stress. Once the model is established, it will be used to test the hypothesis of the synergistic action of antioxidant and glucocorticoid drugs.
4.1. Introduction

It is widely accepted that glucocorticoids are able to suppress the inflammatory response, one of its primary approaches being their ability to switch off multiple activated inflammatory genes that encode cytokines, chemokines, adhesion molecules, and inflammatory enzymes. These genes are usually not ready for transcription until they are released from the attached histones octamer. This release is catalyzed by histone acetyltransferase (HAT) [144]. Pro-inflammatory factors induce the production of NF-kappaB, which activates expression of HAT and thus directly stimulates the expression of inflammatory genes. Once glucocorticoid binds to its receptor and translocates to the nucleus, it activates expression of histone deacetylase-2 (HDAC2), which counters action of HAT and reverses the process of expression of pro-inflammatory genes. However, in COPD, exogenous oxidative stress impairs the translocation of the glucocorticoid receptor and the activity of HDAC2 through the nitrosylation, oxidation and phosphorylation of various factors, leading to the phenomenon known as glucocorticoid resistance [145].

Given that ROS plays a role as a pro-inflammatory factor, enzymatic antioxidants and enzyme mimetics have been selectively applied in the treatment of various inflammatory diseases.
For example SOD, the most widely used enzymatic anti-oxidant, has been found to have an anti-inflammatory effect in myocardial ischemia, reperfusion injury, arthritis, and secondary spinal injury [146] [147]. Many studies have shown the benefit of its administration, and one study demonstrating that transgenic mice with another type of SOD (human Gu/Zn SOD) were found to become resistant to an elastase- or cigarette smoking-induced COPD model, while the SOD gene knocked out mice were found to be highly sensitive in the same model.

SOD mimetics may have several advantages over the native enzyme, including increased stability, low molecular weight, better permeability through various barriers, and lack of immunogenicity [148]. SOD mimetics also appear to have potential in treating inflammatory caused by COPD [149]. Tanka et al. found that not only intravenous administration but also inhalation of PC-SOD ameliorates pulmonary inflammatory, emphysema, and dysfunction through the suppression of cell death, a decrease in proteases, the reduction of the expression of pro-inflammatory cytokines and chemokine, and an increase in the level of antiprotease [106]. Since the anti-inflammatory effect of glucocorticoid is resisted by ROS, in addition to acting as an anti-inflammatory drug directly, antioxidants are anticipated to restore the anti-inflammatory function of glucocorticoids. The synergy effect is expected to result from the administration of glucocorticoids and antioxidants simultaneously.

Nanotechnology has recently been exploited to enhance the pharmacokinetics properties and
therapeutic index of various drugs. A large number of nano-carriers have been developed and tested. Among these, polymeric nanoparticles (NPs) and liposomes represent two most studied classes.

Application of nano-sized drug delivery systems in pulmonary drug delivery offer several special advantages, including the possibility to achieve the uniform distribution in the alveoli. In earlier studies, the suspension of nano-carriers was nebulized for inhalation. However, because of the instability of drugs in solution and the aggregation caused by introduced energy during nebulization, recent researchers preferred to lyophilize nano-carriers and administrate them by dry powder inhaler (DPI).

Nanoparticulate carriers are capable of simultaneous delivery of two or more drugs, ensuring all drugs are functional at the same location. Compared to deliver drugs separately, co-delivery of multiple drugs has several potential advantages, including: 1) ensuring synergistic effects, 2) simultaneously and the same place release, and 3) tenability of relative dosage of various drugs. For example, many gene delivery systems have incorporated another drug to facilitate the gene expression. However, in most cases, either two hydrophobic or two hydrophilic drugs were loaded by the same approaches, for example emulsion and encapsulation. In this study, we planned to design an appropriate nano-carriers to load both the antioxidants (SOD or SOD mimetic) and glucocorticoids (dexamethasone) simultaneously.
4.2. Materials and methods

4.2.1. Materials

HEPES buffer (#BDH4518) was purchased from VWR (West Chester, PA). AlexaFluor®594 (#A20004) fluorescent dye and octadecyl rhodamine B chloride R18 (#0246) were purchased from Invitrogen Life Technologies Corp. (Carlsbad, CA). Cross-linker Traut's reagent (2-iminothiolane·HCl) (#26101) was purchased from Thermo Scientific (Waltham, MA). SOD from bovine liver (3000 u/mg, #574594) was obtained from Calbiochem (Gibbstown, NJ). The SOD assay kit (#19160-1kF) was purchased from Sigma-Aldrich (St. Louis, MO). According to the manufacturer’s information sheet, anti-NR1 antibody is specific to synthetic peptide corresponding to the C-terminal region of rat NMDAR1 (amino acids 918-938). This sequence is identical in mouse NR1, human NR1 (short and long forms), and rat NR1 isoforms NR1b, NR1c, and NR1f. L-a-phosphatidylcholine, hydrogenated (Soy) (#840058), cholesterol (#700100), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(Polyethylene glycol)-2000) (mPEG2000-DSPE, #880120), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimide(polyethylene glycol)-2000) (MAL-PEG2000-DSPE, #880126) were purchased from Avanti Polar Lipid Inc. (Alabaster, AL). Poly (DL-lactide) (#531162, inherent viscosity 0.55-0.75 dl/g, MW 75,000-120,000),
poly(L-lactide)-co-Poly(ethylene glycol) (#P2066, lactide: glycolide ratio 65:35, Mw 40,000-75,000), and rabbit serum Albumin were purchased from Sigma-Aldrich (St. Louis, MO). Dextran 70 (#D1449, Mw 70,000) was purchased from TCI America (Portland, OR). Chloroform, acetone and DMF were purchased from Calbiochem (Gibbstown, NJ). All other chemicals were purchased from Sigma-Aldrich and were used without further purification. D(+) -sucrose and D(+) -trehalose dehydrate were purchased from TCI.

4.2.2. Preparation of Lipid–Polymer Hybrid Nanoparticles

Conventional PLA-PEG NPs were prepared using the self-assembly method described by Cheng et al. [150]. Briefly, the PLA-co-PEG and PLA polymer blend were prepared by dissolving 160 mg of PLA and 40 mg of PLA-PEG co-polymer in 16 ml acetone, and 10 μg octadecyl rhodamine B (10 mg/mL in DMF) was added to this solution if label is needed. Later this organic phase was gently added to 160 mL HPLC grade water in a glass beaker, agitated by ultra-sonication (5510 Branson® Tabletop cleaner, Branson). NPs began to form due to the precipitation in water. After 45 minutes of ultra-sonication, the PLA-PEG NPs were transferred into centrifuge tubes and to remove excess reagents through repeated centrifugation (4500g, 0.5 h; Allegra® 64R centrifuge, Beckman Coulter® USA). Specifically, the supernatant was removed after each centrifugation round, and the NPs were re-suspended in the same volume of HPLC-grade water. Finally, the purified NPs were concentrated into in 4 mL HEPES buffer (20mM, pH 8.5).
For the preparation of the lipid–polymer hybrid NPs, 40mg of MAL-PEG2000-DSPE, an artificial lipid-polymer, was used instead of the PLA-PEG co-polymer; these lipid–polymer hybrid NPs later followed the same purification procedure as described above.

4.2.3. Preparation of the Liposomes

Liposome consisting PC: Chol: DSPE-PEG2000: DSPE-PEG2000-MAL at 55: 39: 4: 2 (molar ratio) were prepared by the thin film hydration method as previously described. Briefly, lipid components were mixed and dissolved in chloroform and dried through rotary evaporation, forming a thin lipid layer. Later, mixed lipid rehydrated by HEPEA buffer, and ultra-sonicated using a probe sonicator (Omni Ruptor 4000, Kennesaw, USA) at 200 W output for 2 min. Total lipid concentration consisted of 10 mg/mL.

4.2.4. SOD Conjugation to Nanoparticles and Liposomes

SOD was loaded on lipid–polymer hybrid NPs and liposomes, using the reaction between the maleimide and the thiol group. However, since SOD from bovine does not have an available thiol group, Traut’s reagent (2-Iminothiolane·HCl) was used to modify the SOD, adding thiol groups. SOD was dissolved in mL HEPES buffer with 1 mg/mL (20mM, pH 6.5), while Traut’s reagent was also dissolved in HEPES buffer (2 mg/mL) and then added to this SOD solution with a 20:1 molar ratio. This mixture was kept in an incubator-shaker for 1 hour (280 rpm, room temperature). After incubation, unlinked Traut’s reagent was removed through centrifugation using a microsep (10
KDa MWCO). Concentrated modified SOD remaining on the top of the microsep centrifuging device was collected and suspended in HEPES buffer, followed by multiple membrane washings with HEPES.

For lipid–polymer hybrid NPs and liposomes, the modified SOD solution was added to the suspension of the nano-carriers. Samples were then incubated overnight at room temperature. For the NPs, modified SOD molecules that were not binding to the nano-carriers were removed using repeated centrifugation (4500g, 30 min). For the liposomes, dialysis was used to remove the non-bounded SOD molecules using Biotech Cellulose Dialysis Membranes (MWCO: 300,000, Diameter: 10mm Spectrum). Dialysis membrane tube containing liposomes and with jaw pinchcocks on both ends were immersed in a 1L of 20 mM HEPES buffer for 3 day dialysis. Dialysis buffer was changed every 8 hours.

4.2.5. Bulk-loaded SOD-PLGA Nanoparticles

Bulk-loaded SOD-PLGA NPs were formulated using a double emulsion solvent evaporation technique as described by Reddy et al. [138, 151]. Using a typical formulation procedure, PLGA polymer was dissolved in chloroform (20 mg/mL), while an aqueous solution of proteins was prepared by dissolving SOD in the HEPES buffer along with RSA (40 mg/mL of both SOD and RSA). This mixture with 10:1 oil to water ratio was emulsified, forming a water-in-oil colloid system through ultra-sonicating by a probe homogenizer (Omni Ruptor 4000, Kennesaw, GA) at 30
W output. This water-in-oil emulsion was emulsified in a 5% w/v PVA solution also through ultra-sonicating with 10:1 water to oil ratio. The emulsion was agitated overnight in an open centrifugation tube in the chemical hood at room temperature to evaporate the chloroform. The PVA polymer, un-incorporated SOD, and RSA were removed through repeated centrifugation (4500g, 30 min). Finally, the suspension was reconstituted into HEPS buffer.

**4.2.6. SOD mimetic encapsulation into liposomes and PLGA nanoparticles**

MnTMPyP, a metalloporphyrin-based superoxide dismutase/catalase mimetic, is a hydrophilic small molecule. Unlike SOD, SOD mimetics lack functional groups that can be used as the reaction site for cross-linking. Thus, physical encapsulation was therefore applied to load the SOD mimetic. The lipid cake after rotary evaporation was ultra-sonicated in the SOD mimetic solution, and therefore, during the formation of Lipid bilayer, a portion of SOD mimetic was encapsulated inside the liposomes. Non-encapsulated SOD mimetics were removed via dialysis.

For PLGA nanoparticles, the encapsulation procedure was the same as SOD, the double emulsion method was applied, and centrifugation was used to remove non-encapsulated SOD mimetics.

**4.2.7. DEX incorporation in the hydrophobic structure of nano-carriers**

DEX is a hydrophobic glucocorticoid. To achieve incorporation into PLA nanoparticles, it was
dissolved in acetone along with PLA. Due to its hydrophobicity, DEX would co-precipitate along with the polymer. In the case of liposomes, DEX was added to the lipid solution in chloroform before rotary evaporation. During ultra-sonication, similarly to cholesterol, DEX would be incorporated into the lipid bilayer.

4.2.8. Antibody Conjugation to Nanoparticles and Liposomes

As described previously, antibodies were modified using Traut’s reagent before conjugation to lipid-polymer hybrid NPs or liposomes; for the bulk-loaded PLGA NPs, Diazirine crosslinkers were used.

4.2.9. Analysis of SOD Activity

SOD activity was measured using an SOD assay kit (Sigma-Aldrich® Corporate life science), which uses a water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophosphene)-5-(2,4-disulfophenyl)-2H-terazolium monosodium) to produce a water soluble formazan dye (WST-1 formazan) upon interaction with superoxide. Superoxide is generated by the xanthine oxidase/ xanthine system. The rate of the production of the WST-1 formazan dye is linearly related to the concentration of the in situ generated superoxide, which is reduced by SOD. The colorimetric method is utilized for detection since WST-1 is colorless while WST-1 formazan has a characteristic absorption maximum of 450nm.
A 20 μl aliquot of each sample (n=3) was added to a 96-well plate, followed by the addition of the assay reagents. The plate was incubated at 37°C for 40 min, and the absorbance at 450nm was read using a microplate reader (Bio-Tek Instruments, Inc.). The standard plot (from 0.1 to 100 U/ml of SOD) was prepared by diluting known SOD concentrations in the dilution buffer provided by the assay manufacturer. The inhibition rate was calculated using the following equation:

\[
\text{Inhibition rate } \% = \left( \frac{(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{blank 1}} - A_{\text{blank 3}})} \right) \times 100\%
\]

Since the most accurate determination of SOD activity occurs for SOD concentrations between 1 and 20 U/ml, all samples with unknown SOD activity were diluted prior to conducting the assay to ensure that their activity was within this range. To measure the total amount of incorporated SOD excluding the influence from the polymeric shell for PLGA bulk-loaded NPs, chloroform was used to dissolve the polymer after lyophilization. Then the immiscible SOD was extracted from the chloroform using water and measured as above.

4.2.10. Characterization of the Prepared Nanoparticles and Liposomes

The hydrodynamic diameters of the NPs and liposomes were measured using dynamic light scattering (DLS). A transparent cuvette was filled with 3 mL of HPLC grade water followed by adding 1 μL of the sample. The suspension was carefully mixed using a pipette with special attention being paid to avoid the formation of bubbles. Capped cuvette was placed in a 90 Plus
particle size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) and DLS data were read for 30 min (10 runs, 3 min per run). Zeta-potential was also measured using the 90 Plus particle size analyzer with the electrode immersed in the suspension.

### 4.2.11. Lyophilization and Accelerated Aging Experiments

Lyophilization is a standard approach used to protect nano-carriers from degradation. Sucrose and trehalose were applied as cryoprotectors. The common concentration of cryoprotectors is 10% (w/v), and the 10X solutions were prepared in advance for storage. This concentrated cryoprotector solution was added to the sample to achieve 10% concentration. Samples were frozen overnight at -80 °C before being lyophilized for 48 hrs.

To shorten the duration of the aging experiment, 37 °C incubation was utilized to achieve an aging rate of about 3 times faster than at room temperature. Although higher temperatures, for example 50 °C, could be used for other materials, these are not suitable for PLA and lipid, because, the Tg for PLA is approximate 40 °C, changing slightly based on polymer molecular weight, and lipid tends to degrade above 40 °C.

To detect the relationship between the SOD activity and its structure, circular dichroism (CD) spectrum was used to analyze the structural change of SOD on liposomes during incubation at 37 °C. CD spectra of SOD, estimated the percentage of α-helix, β-sheet, and other coils. Structural information was collected at times (APPENDIX).
4.2.12. In vitro drug release profile

Typically, the drug release profile of water-soluble molecules, such as SOD and SOD mimetic in this study is performed in a physiological buffer solution, such as PBS. However, the water insoluble molecules, such as DEX were not suitable for this method. One way to determine the release profile is using a solvent that can dissolve the drug. However, it is not the way that the drugs are released in the physiological situation; furthermore, most of such solvents also dissolve the nano-carriers.

In the physiological situation, proteins, such as albumin bind to hydrophobic molecules and facilitate their transportation. Thus, we proposed a novel method to study in vitro release using dialysis against 3 wt. % bovine serum albumin (BSA) in PBS buffer, mimicking the native physiological conditions.

Liposomes with DEX were placed in 300 kDa dialysis tubes and dialyzed against 3% BSA in PBS buffer. DEX concentration in the tubes was determined at different time point. The DEX concentration can be assayed by absorbance at 283nm (the absorbance spectrum peak for estrogens [152]). We determined DEX concentration at 0, 1, 2, 3, 5, 8 and 12 days. This experiment illustrates the release profile of a hydrophobic water insoluble drug from the liposomes.
4.3. Results and discussion

4.3.1. Single drug loading

Because of the advantages of nano-devices in drug delivery, they have already been explored for delivery of SOD for various pathological conditions. Reddy’s group, for example, has studied poly (lactide-co-glycolide) (PLGA) NPs loaded with SOD to protect human neurons against hydrogen peroxide *in vitro* [138, 151]. They used a water/oil/water double emulsion method to form hollow PLGA nano-shell which entrapped 75% of the initial protein into NPs, yielding 90 μL of SOD per mg of NPs, equivalent to 400 U.

In addition to polymeric NPs, liposomes have also been used to deliver SOD, the results indicating that they prolonged the circulation time. For example, Corvo et al. studied SOD entrapped in liposomes with an extended period of the *in vivo* circulating using the dehydration-rehydration method followed by extrusion [101].

All these studies with SOD encapsulated into nano-carriers showed promising result in drug delivery. However, the therapeutic activity of these carriers may be limited by the rate of release of the enzyme from nano-carriers at the site of action. Extensive research indicates that complete degradation of PLA and PLGA NPs takes days to weeks; it takes hours to days to release most of the incorporated drug in vitro, highly depending on the composition, size, and molecular weight (Mw) of the polymer, and the properties of the incorporated drug [153-155]. On the contrary, the
circulation time of these nano-carriers in the blood varies from minutes to hours after administration, also depending on the size, composition, and Mw.

Because of these issues, Gaspar. et al. studied enzymosomes with surface-exposed superoxide dismutase, which are PEGylated liposomes conjugated with acylated superoxide dismutase (ac-SOD), expected to be more beneficial than encapsulated SOD [156, 157]. However, palmitoyl chloride was applied to modify SOD to achieve covalent bonding to the liposome may significantly alter the hydrophobicity of the SOD and then decrease its activity. In addition, Hu et al. have developed novel lipid-polymer hybrid nanoparticles exposing maleimide groups on the surface of NPs which can react with cysteine of proteins. These maleimides, however, were designed to immobilize targeting reagents originally rather than protein drugs, and lipid component with maleimide can only be applied in NPs prepared through self-assembly approach [158].

In this study, the SOD was immobilized either on the surface of the PLA NPs and liposomes or encapsulated inside double-emulsion PLGA NPs, while the SOD mimetic was loaded inside the double-emulsion PLGA NPs or liposomes. Hydrophobic DEX was always incorporated into the bulk of the nanoparticles or into the lipid bilayer of liposomes.

The SOD was first loaded on the surface of the liposomes by adding lipid-maleimide into the liposome structure forming the available binding sites on the surface of liposomes and using a cross-linker to form the covalent bond. With 2 mg/mL of 5000 u/mg of SOD added to the
liposomes, the loading capacity was around 2000 U/mL (1930±575 U/mL) (Figure 6a). Subsequently, the double-emulsion method was also investigated. Since the SOD solution was encapsulated inside the PLGA double-emulsion NPs, its available SOD activity from the intact PLGA NPs was low (205±50 U/mL). However, a much higher SOD activity was detectable after the polymer shell was dissolved using an organic solvent. Total SOD activity measured after removal of the polymeric shell was found to be 4140±1140 U/mL, however exhibiting a significant variation (Figure 6a).

In addition, the same maleimide-terminated PEG-lipid was applied to modify the solid PLA NPs forming a hybrid nanoparticle system. However, relatively low final SOD activity was achieved (1040±176 U/mL) with the same initial concentration of SOD (Figure 6a). A further experiment found that the final SOD activity was primarily based on the amount of the available reactive moiety (the maleimide group) on the hybrid NPs or liposomes. Better coating by maleimide-terminated PEG-lipid on the surface of the liposomes compared to the hybrid NPs led to much higher SOD loading.

The hydrophilic SOD mimetic was encapsulated in both liposomes and double-emulsion NPs. With the same initial activity, the loading capacity of the SOD mimetic for the PLGA double-emulsion nano-carriers was much lower than for liposomes (3210±130 U/mL vs. 1310±450 U/mL), and the loading capacity of the double-emulsion NPs also exhibited more
variation (Figure 6b). Such high variability is consistent with the complexity of the preparation of double emulsion NPs, which includes two steps of ultra-sonication and chloroform evaporation step, which inducing some undesirable leakage.

Similarly to bulk loaded SOD-PLGA NPs, the majority of SOD activity in the case of SOD mimetic liposomes or SOD mimetic PLGA NPs was not available until the lipid bilayer was broken via ultra-sonication or the polymeric shell was degraded by an organic solvent.
Figure 6. Drug loading of several nano-carrier systems with a single drug. A. SOD was covalently immobilized on liposomes and solid PLA NPs. Highest SOD activity was found on intact SOD-liposomes. The total activity in the case of double-emulsion PLGA NPs was much greater, but most SOD activity was not available until PLGA polymer shell was degraded by organic solvent. B. SOD mimetic was encapsulated in both liposomes and double-emulsion PLGA NPs, and liposomes possessed markedly
higher SOD activity. In addition, similarly to encapsulated SOD, most SOD mimetic activity was not available until destruction of the nano-carrier. C. DEX was incorporated into the lipid bilayer of the liposomes or co-precipitated in solid PLA NPs, giving a similar drug loading.

DEX was loaded either into the core of PLA NPs or into the lipid bilayer of liposomes. Too much DEX led to limited loading yield and presence of large DEX crystals in the suspension detectable using optical microscopy. From preliminary studies we found that the initial DEX used for the preparation of either PLA NPs or liposomes was not to exceed 5 mg/mL, resulting in a loading yield of less around 30% in both cases (Figure 6c).

Table 2 summarizes the properties of the nano-carriers with single drug loading. Plain nano-carrier samples were also included as controls. As shown in the table, liposomes were much smaller than NPs. Previous research demonstrated that longer ultra-sonication leads to the smaller size of the liposomes [159]. Meanwhile, the size of the PLA NPs was mainly dependent on the molecular weight of the polymer. On the other hand, the size of double-emulsion PLGA NPs was much larger than that of the liposomes or the hybrid NPs. Zeta-potentials were also measured, as a characteristic of the stability of colloidal dispersions. As showed in Table 2, all had highly negative zeta-potential below -40 mV, sufficient to ensure high stability of the colloidal system.
There was no obvious change in either size or zeta-potential upon conjugation of SOD on the surface of a nano-carrier.

Table 2. Summary of characteristics of nano-carriers both before and after the loading antioxidants or glucocorticoids. The particle size was the number average measured by DLS.

<table>
<thead>
<tr>
<th></th>
<th>Liposomes (n=8)</th>
<th>PLA NPs (n=6)</th>
<th>PLGA NPs (n=4)</th>
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<tr>
<td>Bare SOD</td>
<td>123.8 ±25.0</td>
<td>199.1 N/A</td>
<td>202.9 N/A</td>
</tr>
<tr>
<td>SOD mimic</td>
<td>127.9 ±19.1</td>
<td>198.8 ±29.2</td>
<td>242.3 ±63.2</td>
</tr>
<tr>
<td>DEX</td>
<td>132.0 ±27.7</td>
<td>198.8 ±29.2</td>
<td>257.1 ±63.2</td>
</tr>
<tr>
<td>Size (nm)</td>
<td>133.2 ±44.3</td>
<td>202.9 ±8.2</td>
<td>254.9 ±43.0</td>
</tr>
<tr>
<td></td>
<td>±34.3</td>
<td>±8.2</td>
<td>±38.1</td>
</tr>
<tr>
<td>Zeta-potential (mV)</td>
<td>-43.7 ±5.4</td>
<td>-50.3 ±6.4</td>
<td>-60.3 ±4.6</td>
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<tr>
<td></td>
<td>±49.8</td>
<td>±63.2</td>
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<td>±44.2</td>
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<td>±42.9</td>
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4.3.2. Lyophilization and long-term stability

Lyophilization is necessary for long-term self-storage. Stability of the nano-carriers after lyophilization was evaluated by measuring changes in size, zeta potential, and SOD activity. As
can be seen in Figure 7a, lyophilization resulted in a tremendous reduction of SOD activity in all samples in the absence of cryoprotectors. However, use of cryoprotectors, sucrose and trehalose, allowed us to prevent the loss of activity in all types of nano-carriers leading to retention of more than 75% of the initial activity in all cases. Sucrose provided better protection than trehalose. It was found that double-emulsion NP is much more vulnerable to the loss of activity after lyophilization, even with cryoprotectors.
Figure 7. Stability of SOD-loaded nano-carriers after lyophilization with and without cryoprotectors (sucrose and trehalose). A. SOD activity decreased dramatically after lyophilization without cryoprotectors, but cryoprotectors reduced the loss of activity in all types of nano-carriers. B. Cryoprotectors prevented aggregation in all types of nano-carriers. (*P<0.01, **P< 0.05, *** P <0.001)

In addition to the loss of activity, aggregation after lyophilization could be a concern for drug-loaded nano-carriers. As can be seen from Figure 7b, size of the nano-carriers increases considerably after lyophilization. The use of cryoprotectors allowed us to solve this problem. Little size increase (<10% in all cases) was observed for the samples lyophilized with cryoprotectors, while the size of the liposomes and double-emulsion PLGA NPs in the absence of
cryoprotectors increased by 30% to 40%, a change attributed by partial aggregation. Much more severe aggregation was detected in the case of unprotected lyophilized PLA nano-carriers (830±10%).

The activity of the SOD-coated liposomes after long-term storage at 37 °C is shown in Figure 8. This experiment compared the stability of the liposomes and the PLA NPs stored in colloidal suspension to that of freeze dried samples with a cryoprotector. The samples stored as colloidal suspensions lost more activity than their freeze-dried counterparts for both liposomes and hybrid NPs. The lyophilized liposomes retained 80±3% of the original SOD activity with trehalose, and 83±1% with sucrose after 120 days of incubation at 37°C, which corresponds to a 1 year shelf-life at room temperature. At the same time, liposomes stored in the solution retained only 58±3% of SOD activity (P< 0.05). Similarly, the long-term stability of the hybrid NPs was also higher for samples lyophilized with cryoprotectors. Here, samples with sucrose and trehalose retained 85±2% and 83±5% respectively, while the colloidal suspension retained 73±4% of the initial activity.

4.3.3. Glucocorticoid release profile

Unlike most water-soluble drugs, the transportation of hydrophobic molecules, for example steroids, is facilitated by some proteins. Here, a new dialysis-based technique was established to study the release of poorly soluble hydrophobic molecules from nano-carriers. Serum albumin
was added into the dialysis buffer to facilitate the release of dexamethasone from the liposomes.

As seen in the Figure 9, the release of DEX in the liposomes in a buffer is restricted without BSA because of its poor solubility on water. Free DEX suspension in the absence of liposomes was removed completely from the dialysis tube within 2 days. In contrast, release from the DEX-loaded liposomes was much longer and took more than 8 days.
Figure 8. Long time shelf-life study for both SOD PLA (crosslinked) (A) and SOD liposomes (B). With sucrose and trehalose cryoprotectors, 80% of activity was retained after 120 days of accelerated aging at 37°C, which corresponds to 1 year shelf-life on storage at room temperature. SOD activity immediately after freeze drying was considered to be 100%. Nano-carriers without any cryoprotector (square); SOD liposomes with sucrose (circle) and trehalose (triangle). *P<0.01, **P< 0.05, *** P<0.001
Figure 9. Drug release profile of DEX loaded liposomes. All free DEX sample was released in 2 days, while liposomes resulted into a more continuous and prolonged release. Drug was found not to release in BSA free buffer.

In summary, sucrose performed better than trehalose as the cryoprotector. Hybrid NPs were found to be less stable and more prone to aggregation than liposomes. Double emulsion PLGA NPs showed lower SOD activity and were also found to be somewhat more vulnerable to aggregation and loss of activity upon lyophilization. In addition, all three kinds of the therapeutic molecules, SOD, SOD mimetic, and DEX, can be loaded into liposomes, and liposomes showed better loading capacity of SOD and SOD mimetic compared to the other nano-carriers. Thus, liposomes were selected as the delivery vehicle to load both antioxidant and glucocorticoids.
4.3.4. Simultaneous loading of antioxidant and glucocorticoid

The co-delivery system, loading two therapeutic molecules into one single nano-carrier, reduces the dosage of nano-carriers and ensures their simultaneous release. Here two sets of liposome samples were prepared, one with SOD loaded on the outer surface via covalent binding and DEX loaded into the lipid bilayer, and the other encapsulating SOD mimetic inside the liposome and DEX loaded in the lipid bilayer. As seen in Figure 10, there was no significant decrease of the loading capacity of both the antioxidant and the glucocorticoids in these binary systems. Unlike other co-delivery systems, where two types of therapeutic molecules were loaded via the same mechanism, in this study, antioxidants and glucocorticoid were loaded using two separate steps.

4.4. Conclusion

From the above results, liposomes were selected as the best nano-carrier that can carry both antioxidants and glucocorticoids. Liposomes were more stable upon lyophilization and during the long-term storage and allowed higher loading yield of both therapeutic molecules.
Figure 10. The retained loading capacity in the co-delivery system. Both SOD+DEX (A) and SOD mimetic+DEX (B) loaded liposome systems were prepared, and compared with single drug design, these two systems retained most drug loading capacity.
CHAPTER 5

THE OPTIMIZATION OF NANO-CARRIER PROPERTIES FOR MAXIMUM TARGETING EFFICACY

5.1. Introduction

Currently, nano-sized delivery vehicles are the focus of much research for drug delivery in treatment of various diseases [155, 160, 161]. Therapeutic molecules are packed into nano-sized carriers, for example liposomes and polymeric nanoparticles, to selectively improve their bio-availability, bio-compatibility and safety profiles; prolong circulation time; and reduce toxicity, and with the continued development of techniques for nano-sized vehicle synthesis and research enhancing their ability to address specific areas of need, it is expected that their utility in drug delivery will continue to grow [162-164].

Among these techniques, modification of the surface of nano-sized vehicles to achieve appropriate accumulation at the site of interest while at the same time reducing delivery to the rest of the body has received much interest, because many drugs induce systemic side-effects in normal tissue [165, 166]. Therefore, in addition to wrapping the drug in a well-designed package, its preferential delivery to the site of interest can be expected to reduce systemic side-effects while simultaneously increasing the therapeutic effect. This targeted strategy is a prominent area of drug delivery research [164, 167].
Active targeting is usually achieved by modifying the nano-sized vehicle with the molecules which can bind to cells in the disease site. This binding and following cellular internalization reduces the local concentration of the nano-sized vehicle, and then concentration gradients attract more vehicles via free diffusion [168]. This binding not only facilitates the local accumulation but also assists the internalization of the vehicle into the cells, which is essential for the drug functions intracellularly [169]. Normally, this specific binding was obtained by modifying the surface of the nano-sized vehicle with targeting ligands specifically recognizing disease-associated biomarkers. Antibody (or antibody fragment)/antigen and ligand (or its structural analogues)/receptor couples have been extensively studied for this targeting strategy, owing to their high specificity and high binding affinity [170]. For example, folic acid receptors, overexpressed in several tumor types, act as the pathology-associated biomarkers when drug-loaded nano-sized vehicles are modified by folic acid or its structural analogues [171, 172], while vehicles designed to target endothelial cells expressing the intercellular adhesion molecules (ICAMs) have been targeted using anti-ICAM antibodies [173, 174].

The targeting efficacy of this surface modification, represented by the binding affinity from the addition of a targeting entity through surface modification, has been demonstrated by numerous studies [167, 175, 176]. More recently, several investigations have focused on targeting performance, with the multivalent design having been shown to enhance targeting efficiency [171].
Specifically, simultaneous binding of multiple agents on a single nano-sized vehicle to more than one ligand/receptor leads to a synergistic or additive binding affinity, which is referred to as the avidity effect. This effect has been found to significantly increase the apparent binding affinity, thus raising the targeting efficacy [177, 178]. While this positive correlation between surface density and the targeting efficacy has been widely accepted, some studies observed a surface density saturation phenomenon. These researchers noted that once the surface density of a targeting agent reached certain threshold value, its further increase did not increase the binding affinity; in fact, in some cases it even decreased [179].

However, this relationship between the saturation phenomenon and the amount of targeting agent was primarily investigated for ligand/receptor couples, and the targeting efficacy was analyzed by the cell uptake rate of nano-sized vehicles and was difficult to quantify. In comparison to ligand/receptor couples, antibody-modified nano-carriers have received much less attention, raising the question of whether the saturation phenomenon is present in antibody-based targeting systems. In addition, \textit{in vitro} targeting experiments depend, in part, on cell viability, expression of the receptors by cells in a specific set of \textit{in vitro} conditions, and other cellular characteristics that may introduce additional unknowns in the experiment. Moreover, the specific threshold value of the surface density has not been clearly determined for the saturation phenomenon, something that seems to be crucial for the design of nano-sized vectors and optimization of their targeting ability.
Finally, the relationship between the threshold of the surface density and the properties of delivery vehicles or other factors has not been fully investigated.

Here, we report a quantitative study of the saturation phenomenon of an antibody-modified nano-sized vehicle system. In order to better understand the relationship between the surface density of the targeting antibody and the targeting ability of the vector, we employed Surface Plasmon Resonance (SPR), a powerful label-free method originally developed to study the interaction between macromolecules [180]. Different from most static measurements, SPR is a real-time measurement that allows direct determination of binding parameters such as affinity constants and kinetic rates. SPR has been widely used to characterize the properties of ligand-modified nano-sized vehicles targeting their corresponding receptors [171, 181-183]. While the results of these studies have demonstrated the avidity effect via increasing the surface density of the targeting molecule, it has not been employed to explore the saturation effect. In this study, we use SPR to analyze the saturation phenomena for antibody-coated nanoscale carriers. For this purpose, we used liposomes as a model nano-sized carrier.
5.2. Materials and methods

5.2.1. Materials

Hydrogenated (Soy) (#840058), cholesterol (#700100), 1,2-distearoyl-sn-glycero-3-Phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (mPEG2000-DSPE, #880120), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-(N-maleimide(polyethylene glycol)-2000) (MAL-PEG2000-DSPE, #880126) were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, USA). Dulbecco’s modification of Eagle’s medium (DMEM), antibiotic-antimycotic solution (Anti-Anti), and fetal bovine serum (FBS) regular (Heat Inactivated) were purchased from Corning Cellgro. Kaighn's modification of Ham's F-12 medium (K-F12) was acquired from ATCC. A research-grade CM5 (carboxymethyl dextran) sensor chip was purchased from Biacore AB (Uppsala, Sweden). NHS (N-hydroxysuccinimide), EDC (N-ethyl-N-(3-dimethylamino-propyl)-carbodi-imide hydrochloride), ethanolamine/HCl and other buffer solution reagents were purchased from Sigma-Aldrich. Anti-Glutamate Receptor NMDAR1 (NR1) antibody produced in rabbit. Anti-MUC1 antibody produced in rabbit, Anti-Mouse IgG (whole molecule) antibody produced in goat, and monoclonal anti-hepatocyte growth factor antibody produced in mouse were also purchased from Sigma.

5.2.2. Liposome preparation

PEGylated liposomes were prepared as described in previous studies [184]. The lipids used
were phosphatidylcholine, cholesterol, mPEG2000-DSPE, and MAL-PEG2000-DSPE at the molar ratio of 55:39:4:2. Total lipid concentration consisted of 10 mg/mL. Lipid components were weighted as the molar ratio mentioned above, mixed and dissolved in chloroform (10 mg/mL), and dried through rotatory evaporation, forming a thin lipid layer. These dried lipid films or cakes were removed from the flasks, frozen, and stored. The mixed lipid film was subsequently rehydrated with a HEPES buffer (20mM, pH=6.5) and dispersed using a probe ultra-sonicator (Omni Ruptor 4000, Kennesaw, GA, USA) at 200W output. Different sonication durations were applied to create liposomes with different diameters. For assay requiring cell labeling, octadecyl rhodamine B dye was added to the chloroform mixture right before rotatory evaporation. An ice-water bath was applied to the lipid mixture preventing the heat from sonication, and then a 0.45 μm nylon syringe filter was used to remove the large aggregations of liposomes formed during preparation which reduced the polydispersity index (PDI).

5.2.3. Liposome characterization

Liposomes were diluted in deionized water to approximately 5 μg/mL as recommended in the manual of particle sizer. Their mean particle diameter and the width of the particle distribution (polydispersity index, PDI) were determined through the dynamic light scattering using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). This instrument was also used to quantify the particle charge as zeta-potential.
5.2.4. Targeting ligand conjugation

The introduction of PEG modified with maleimide to the surface of the liposomes offered potential binding sites for peptides or proteins. Maleimide reacts with thiol groups on the cysteines of proteins, forming a stable carbon-sulfur bond, which has been widely applied to immobilize proteins. For a higher loading yield, Traut’s reagent (2-iminothiolane•HCl) was used to modify the protein as it reacts with primary amines (-NH₂) to introduce extra sulfhydryl (-SH) groups while maintaining charge properties similar to the original amino group (Figure 11).

Figure 11. Schematics for modifying liposomes with targeting antibody. (a) Modification of the target antibody with sulphydryl groups. (b) Modified antibodies binding to PEGylated liposomes with maleimide groups.

The conjugation method was described previously [184]. Targeting ligands or control
antibodies were first mixed with Traut’s reagent at 20 molar excess and a final concentration of 0.01 mg/mL in a HEPES buffer (20 mM pH 8.0). After incubation for 1 hour in the dark at room temperature, the unreacted Traut’s reagent was removed by microfiltration using 30 kDa MWCO centrifugal devices. The modified proteins were then mixed with the liposome colloid in the HEPES buffer, 20mM pH 6.5, and incubated overnight at room temperature. Unattached proteins were removed through dialysis with the HEPES buffer (20mM) for 48 hours, replaced every 8 hours using Biotech Cellulose Ester (CE) Dialysis Membranes (MWCO: 300,000, diameter: 10mm).

5.2.5. BCA assay evaluation of targeting ligand surface density

The amount of target ligand immobilized on the liposomes was quantified through microBCA assay. In order to eliminate interference from the lipid, 2% SDS was added to the samples. Traut’s reagent-modified target ligands of known concentrations were included as standard samples. Therefore, the surface density (Ab/μm²) and antibody/liposome ratio (Ab/liposome) were calculated based on the amount of target antibody in addition to the average size of the liposomes and the density of the lipids.
5.2.6. Surface plasmon resonance (SPR)

**Chip preparation**

Quantitative and kinetic interaction of the antibody-modified nanoparticles with the antigen was monitored using the Biacore X system, with data analysis being conducted using BIAevaluation software. CM5 sensor chips were used in each experiment.

Ten mM HEPES, pH 7.4; 150mM NaCl; and 0.05% (vol/vol) tween 20 were used as the SPR running buffer, and a 10mM sodium acetate (pH 4.0) buffer was used as the antigen immobilization buffer. All buffers were filtered through 0.45 μm PTFE filters (Millipore) and sonicated for 30 min. The antigen was immobilized through the following procedure (Figure 12): The chip covered with modified dextran was activated with a solution of 70 μL EDC (75 mg/mL) mixed with NHS (12 mg/mL) at a flow rate of 10 μL/mL for 10 min. Then, the antigen solution in the sodium acetate buffer was injected at a flow rate of 10 μL/min for 5 min. The concentration of antigens and the replication of this injection were varied based on the requirement of antibody density on the chip. The Biacore X instrument divided the sensor chip into two independent flow channels. Both were activated by EDC solution, but one was closed during the antigen injection, serving as a reference channel. Finally, both channels were blocked with ethanolamine (100 nM, 20μL/min), quenching remained active binding sites.
Figure 12. Schematics for modifying chips with antigen. Dextran fibers with carboxylic acid groups on the chip were activated by EDC and NHS, and later form covalent bond with amine groups on protein molecules.

**SPR analysis**

After the primer and normalization steps, samples of liposomes or free antibodies were injected into both the reference and the detection channels at a flow rate of 5 μL/min along with the running buffer. Liposome samples and free antibodies were diluted to create various concentrations, and the analyses were conducted in triplicate to ensure accurate results. The sensorgram produced by the Biacore X was recorded as a series of association (during the injection) and the dissociation (after injection) phases for both the reference and the detection channels. Regeneration of the chip surface was achieved using 3 M MgCl₂ and 0.01% SDS (20 μL/min, 5 sec) in turn at a flow rate of
40 μL/mL after each injection. The experiments were conducted using Biacore Control Software, which automatically interprets the refractive index changes on the surface in resident unit (RU), plotted over time. Then, BIAevaluation software was applied to determine the dissociation and association rates and the binding affinity constant by fitting curves (obtained from the subtraction of the reference channel signal from the detection one) into adsorption models.

**Cell binding assay**

Cells were grown as a monolayer culture in flasks at 37°C and under a 5% CO₂ atmosphere in a DMEM or K-F12 medium with 10% FBS and 1% Anti-Anti. Subsequently, they were passaged into 96 well plates one day before the experiment. After the cells grew to a monolayer in the plates, 4% paraformaldehyde was utilized to fix the cells for 15 min. Then the cells were blocked by a blocking solution (PBS with 1% goat serum albumin) for 30 min. Rhodamine-labeled liposomes, modified with a targeting antibody in a series of dilutions, were incubated with the fixed cells at room temperature for 4 hrs. A PBS buffer with 1% tween 20 was used as a washing buffer, and washing was carried out 5 times after each step of the incubation. The amount of conjugated liposomes on the cells was quantified using a fluorescence microplate reader. The fluorescence intensity from Rhodamine at 575nm (excited at 552nm) was shown to be proportional to the amount of liposome.
5.3. Results and discussion

5.3.1. Liposome preparation

Liposomes were prepared from the PEGylated lipids containing maleimide end groups along with cholesterol using a rehydration and ultra-sonication method. Figure 11 illustrates maleimide chemistry used for modification of the PEG layer of the liposomes. As a result of that process, most maleimide groups would be exposed outward since they are immobilized on the end of the PEG polymer chains and form covalent bonds without any intervening spacer between the PEG and the targeting antibody. These PEGylation liposomes with the lipid composition mimetic that of the FDA-approved liposome drug, Doxil. The modification was less than 5% of the mass with the addition of maleimide.

The resulting liposome sizes ranged from 100-300nm, inversely related to ultra-sonication duration. According to previous studies, cavitation caused by oscillating micro-bubbles produces shear fields. Large liposomes entering these fields form long tubelike appendages that can pinch-off, creating smaller ones [159]. Thus, longer sonication duration results in smaller liposomes. However, the zeta-potential of liposomes of various sizes remains constant. All samples were approximately –45 mV, which was negative enough to maintain the stability of the colloid. The final liposome concentration was found to be approximately 8.2 mg/mL, as determined by gravimetric analysis, which corresponds to a concentration of $10^{12}$–$10^{13}$ liposomes per milliliter.
5.3.2. SPR Measurement

Because of high cost of the primary anti-MUC1 antibody, a model based on binding of a secondary antibody was initially extensively analyzed before experiments with an actual targeting antibody. In this secondary antibody model, anti-mouse IgG antibody produced in goat was utilized as the targeting antibody, and a monoclonal anti-hepatocyte growth factor IgG antibody produced in mice was used as the antigen.

**Chip modification and pre-testing**

According to the standard protocol designed for SPR studies of molecular interactions, the antibody should be immobilized on the chip, while the antigen is running as the analyte along with a running buffer. In addition, maintaining a low surface density of the antibody during chip modification is recommended to avoid possible effect of avidity.

To better imitate cell interactions with targeted nano-carrier standard protocol for molecular interaction experiments has been modified in this work. Specifically, the antigens rather than the antibodies were immobilized on the CM5 sensor chip. The targeted antibody-modified liposomes were injected into the flow channels to imitate flow transport of these nano-sized delivery vehicles. The antigen immobilization was achieved through the EDC-based amide coupling method as described in the CM5 chip protocol. For preliminary research, two different antigen-presenting chips were prepared by varying the initial antigen amount. The CM 5 chip with a low surface
density of antigen was prepared using the diluted antigen solution (10 μg/mL) according to the standard protocol (Figure 13a). As demonstrated, both channels on the chip were activated by EDC in combination with NHS, and then the antigen was immobilized on the detection channel, resulting in the increase of approximately 500 resident unit (RU). Based on the instrument design, 1000 RU is equal to 1 ng/mm² protein; therefore 500 RU was equivalent to 0.5 ng/mm² or approximately 2×10⁹ antigens/mm². From the literature, typical density of receptors on the cell surface is much higher, in the range of 10¹⁰ antigens/mm² or more [185] [186] [187]. To prepare a surface with a higher surface density of the antigen, repeated injections of a higher concentration antigen solution (200 μg/mL) were applied. A subsequent washing step removed any non-covalent linked antigens (Figure 13b). In contrast to the earlier low surface density chip, the increase of 6000 RU was achieved, equivalent to 6 ng/mm² or 2.4×10¹⁰ antigens/mm².

Both sensor chips prepared were assessed for binding specificity through the injection of free targeted antibodies (Figure 13c). The curves shown in Figure 13c refer to a mean value obtained from multiple independent measurements (n=3). With a consistent free antibody concentration, various surface densities lead to different adsorption levels and kinetic parameters. Here, both association and dissociation rates (Ka and Kd) were calculated through curve fitting analysis to a simple 1:1 adsorption model (Langmuir model). The association constant (KA), representing the binding affinity of the molecules to the surface, was calculated as KA=Ka/Kd. The detailed
kinetic parameters and the affinity constants for the chips with different surface density of the antigen are listed in Table 3. As expected, free antibody bound stronger to the chip surfaces with high antigen density. This increase of affinity constant was due to the effect of avidity. High density on the chip increased the possibility of forming multiple binding sites between the antibody and the chip since each antibody has two binding sites (Figure 13d). As illustrated in Table 3, the increase of the binding affinity for the high surface density of the antigen was caused by both the increase in the association rate and the decrease in the dissociation rate independently. The decrease in the dissociation rate ((k_d low)/(k_d high)≈10) was much greater than the increase in the association rate ((k_a high)/ (k_a low)≈3), suggesting that the avidity effect, and hence the increased binding affinity, depended more on the slowed dissociation step, possibly because of the much lower probability of the simultaneous detachment of both binding sites [182]. By analogy, liposomes modified by multiple targeting antibody molecules are expected to exhibit much higher binding affinity, primarily because of the significantly slower dissociation process.

Rmax, representing the theoretical maximum signal for each single run, was extrapolated using BIAevaluation software. As shown in Table 3, the ~12-fold increase in the surface density of the antigen resulted in only approximately a 3-fold increase of the Rmax for free antibody binding. Thus, there are multiple unoccupied binding sites on the surface; formation of multiple bonds between the antibody and antigen also could be a contribution (Figure 13d).
Considering the surface density of more than $10^{10}$ molecules per mm$^2$ for a typical target ligand, such as folate receptors on cancer cells, NMDA receptors on neurons, or Epithelial membrane antigen (MUC1) on the surface of epithelial cells [188] [189], the chip with the high antigen concentration was deemed to be more appropriate for further investigations.
Figure 13. CM 5 sensor chip preparations with various antigen densities and pretest with free antibody samples. (a) Sensor chip with low density antigen was prepared by one-time diluted solution injection. (b) Sensor chip with high density was prepared by repeated concentrated solution. (c) Both sensor chips were tested through the free targeting antibody solution of the same concentration. (d) Schematic of the free antibody immobilization on sensor chips both high and low density surfaces.
Table 3. Kinetic parameters and constants of free antibody binding to both low and high density antigens on the sensor chip surface.

<table>
<thead>
<tr>
<th>Chip surface</th>
<th>Rmax (RU)</th>
<th>$k_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$K_A$ (1/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low surface density</td>
<td>1.9 (±0.4)×10³</td>
<td>2.4 (±0.7)×10⁴</td>
<td>3.7 (±0.6)×10⁻⁴</td>
<td>6.5 (±2.0)×10⁷</td>
</tr>
<tr>
<td>High surface density</td>
<td>6.0 (±1.0)×10³</td>
<td>8.0 (±1.8)×10⁴</td>
<td>3.4 (±0.4)×10⁻⁵</td>
<td>2.4 (±0.6)×10⁹</td>
</tr>
</tbody>
</table>

**Surface density of antibodies on liposomes**

Liposome samples (130nm), with a range from 4 to 14 molecules per liposome particle, were prepared by manipulating the initial antibody concentration during liposome formation (Table 4). The final antibody amount per liposome particle was assessed through microBCA assay. As the table illustrates, the maximum antibody loading of this liposome composition was approximately 14 antibody molecules per vehicle. The liposome samples with no antibody modification or with a non-targeted antibody (anti-bovine antibody) served as the control that was tested in advance, the results indicating that the nonspecific binding to the chip surface on plain liposomes and on the liposomes with the control antibody was negligible (APPENDIX).

Antibody-amount dependent binding sensorgrams were acquired by running these samples through the chip with a high antigen surface density (Figure 14a). As shown in the figure, the
dissociation step was much slower than that for the free antibody, and thus a prolonged dissociation was used to enhance the accuracy of the fitting. The dissociation phase lasted more than 20 min for every injection (not fully demonstrated in the figure). Previous research has found the Langmuir model to be appropriate for evaluating the targeting efficacy of antibody modified delivery vehicles. The binding affinity calculated from the curve fitting is shown in Figure 14b, and more detailed kinetic results are shown in Table 4. As illustrated by the figure and the table, from low targeting antibody surface density on liposomes to high, the binding affinity increased significantly at low surface coverage, from \((0.6 \pm 0.2) \times 10^{10} \text{M}^{-1}\) at 3.8 antibodies per liposome to \((2.3 \pm 0.3) \times 10^{10} \text{M}^{-1}\) at 5.8 antibodies per liposome, but later it reached a plateau at ~9 targeting antibodies per liposome particle, or \(1.5 \times 10^8 \text{antibodies/mm}^2\). After that point, further addition of the targeting antibody on the liposomes did not significantly increase the binding affinity \((6.0 \pm 1.3) \times 10^{10} \text{M}^{-1}\) at 13.5 antibodies per liposome vs. \((1.6) \times 10^{10} \text{M}^{-1}\) at 9.2 antibodies per liposome). The saturated binding affinity was found to be approximately \(~6.0 \times 10^{10} \text{M}^{-1}\). This result has shown that, similarly to the conventional ligand/receptor targeting, this targeted nano-carrier/antigen system also met the saturation point at certain antibody coverage, and the apparent binding affinity remained stable with further increase of the antibody surface density.

Similarly to the free antibody, the increase in binding affinity from low to high targeting antibody density was attributed to the reduction of the dissociation rate and the increase in the
association rate. Both kinetic rates also reached a plateau at some point ($\sim 24 \times 10^4 \text{ (Ms)}^{-1}$ for $k_a$ and $4.2 \times 10^6 \text{ (s)}^{-1}$ for $k_d$) (Table 4).

The association rate of liposomes with a low targeting antibody surface density ($(3.9 \pm 0.6) \times 10^4 \text{ (Ms)}^{-1}$ at 3.8 antibodies per liposome) was somewhat lower than that for the free antibody ($(8.0 \pm 1.8) \times 10^4 \text{ (Ms)}^{-1}$). The reason for this result might be much larger size of the liposomes compared with the free antibody. It is known that the association rate is partially determined by its diffusion rate, which is negatively related to its size. However, with the further increase in the amount of antibody on the liposome, the avidity effect suppressed the negative impact from the increased size. On the other hand, compared to the free antibody, the dissociation rate, which contributes the most to the rise in the binding affinity, of the antibody-modified liposomes was reduced by an order of magnitude. This trend further inferred that the increase of association constant, derived from multivalent binding, was mainly contributed by the significantly reduced dissociation rate, meaning that the avidity effect was primarily from the lower probability of simultaneous dissociation of two or more bonds.

Table 4. Characteristics and targeting efficacy of liposomes (130 nm) with various targeting antibody surface densities.
A parameter, $\beta$, was used to represent the increase of the apparent binding affinity from the avidity effect. In this case, $\beta$ for the sample with saturated targeting antibody was approximately 23. Different from most previous studies, owing to the technique of SPR, the association constant was quantitatively determined, which not only enabled the comparison between different samples, but also with free antibody’s association constant. In the future, this approach can also be used to compare different delivery systems and will be helpful to further investigations of the avidity effect.
Figure 14. (a) Sensorgrams of liposomes (130nm) with various target antibody surface densities. To precisely determine the dissociation rate, a prolonged dissociation phase was conducted up to 20 min, but was not fully demonstrated. (b) A plot of binding
affinity as a function of target antibody surface density. Surface density was interpreted in two ways: antibody per liposome ratio, and antibody per millimeter square.

As shown in Table 4, liposomes with a high targeting antibody surface density resulted in a much higher Rmax than liposomes with low surface density of the antibody. Since the binding sites on the sensor chip were abundant, the steric occupation of the surface was the primary limitation for the theoretical maximum signal. Liposomes were negatively charged to repulse each other, maintaining stability in the colloid. This repulsion decreased the bulk density of liposomes on the surface, leading to a low Rmax for a low targeting antibody surface density. However, liposomes with a high targeting antibody density exhibited a much higher Rmax, up to 50% of the chip surface. This increased Rmax could be associated with high avidity, which forces liposomes to pack much closer onto the chip surface.

**Size of liposome**

The diameter of liposomes and other nano-sized vehicles is a crucial parameter in pharmacokinetics as their size provides the potential for crossing the various biological barriers in the body. However, limited research has focused on the effect of the size of a nano-carrier on its targeting ability. Here, we first prepared much larger liposomes (280nm) by reducing the sonication duration. Similarly to the smaller liposomes used in the experiment described above
(130nm), a series of samples with various targeting antibody surface densities was prepared using a similar procedure. With a much larger surface area but a consistent maleimide content in the liposome composition, larger liposomes with more targeting antibodies per liposome (up to 73 antibodies per liposome) were available (Table 5). Similarly to the smaller liposomes, the changes in the SPR sensorgram were observed with the increase of the surface density of the targeting antibody, and saturation was reached at some threshold surface density as indicated by the overlapping kinetic curves in

Figure 15a. Both the positive correlation and the saturation are illustrated in the binding affinity plot:

Figure 15b) and more detailed parameters are shown in Table 5: initially, as the surface density of targeting antibodies increases, the binding affinity increased quite dramatically ((0.3 ±0.1)×10^{11} M^{-1} at 10.7 antibodies per liposome vs. (1.2 ±0.3)×10^{11} M^{-1} at 21.6 antibodies per liposome), and as the amount of the targeting antibody increased, the increase in the binding affinity reached saturation at ~40 antibodies per liposome with the saturating binding affinity of ~2.2×10^{11} M^{-1}.

The threshold of the targeting antibody amount per liposome for larger liposomes was much larger than that for the smaller liposomes (~40 antibodies per liposome vs. ~9 antibodies per liposome, respectively). However, saturation occurs at approximately the same antibody surface
density of $\sim 1.5 \times 10^8$ antibodies/mm$^2$, independently of the size of the liposome. These results indicate that the surface density (antibodies/mm$^2$) rather than number of antibody molecules per liposome determines the saturation of the targeting affinity of these particular nano-sized delivery vectors.

Table 5. Characteristics and targeting efficacy of liposomes (280 nm) with various targeting antibody surface densities.

<table>
<thead>
<tr>
<th>Initial amount (ug/mL)</th>
<th>Loading yield (%)</th>
<th>Antibody per liposome</th>
<th>Antibody Per mm$^2$</th>
<th>Rmax (RU)</th>
<th>$k_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip (280, 10.7)</td>
<td>12.5</td>
<td>94.2 ($\pm 15.6$)</td>
<td>10.7 ($\pm 1.8$)</td>
<td>$0.4 (\pm 0.1) \times 10^8$</td>
<td>$0.7 (\pm 0.2) \times 10^8$</td>
<td>$4.9 (\pm 1.0) \times 10^8$</td>
</tr>
<tr>
<td>Lip (280, 21.6)</td>
<td>25</td>
<td>95.6 ($\pm 9.2$)</td>
<td>21.6 ($\pm 2.0$)</td>
<td>$0.8 (\pm 0.1) \times 10^8$</td>
<td>$1.1 (\pm 0.3) \times 10^8$</td>
<td>$16.9 (\pm 3.2) \times 10^8$</td>
</tr>
<tr>
<td>Lip (280, 29.3)</td>
<td>50</td>
<td>64.8 ($\pm 2.8$)</td>
<td>29.3 ($\pm 1.2$)</td>
<td>$1.2 (\pm 0.1) \times 10^8$</td>
<td>$1.7 (\pm 0.4) \times 10^8$</td>
<td>$19.9 (\pm 3.2) \times 10^8$</td>
</tr>
<tr>
<td>Lip (280, 43.5)</td>
<td>100</td>
<td>48.0 ($\pm 5.7$)</td>
<td>43.5 ($\pm 5.2$)</td>
<td>$1.8 (\pm 0.2) \times 10^8$</td>
<td>$3.0 (\pm 0.6) \times 10^8$</td>
<td>$29.7 (\pm 4.2) \times 10^8$</td>
</tr>
<tr>
<td>Lip (280, 71.0)</td>
<td>150</td>
<td>52.3 ($\pm 1.9$)</td>
<td>71.0 ($\pm 2.6$)</td>
<td>$2.9 (\pm 0.1) \times 10^8$</td>
<td>$3.1 (\pm 0.6) \times 10^8$</td>
<td>$26.7 (\pm 3.3) \times 10^8$</td>
</tr>
<tr>
<td>Lip (280, 73.4)</td>
<td>200</td>
<td>40.5 ($\pm 2.6$)</td>
<td>73.4 ($\pm 4.8$)</td>
<td>$3.0 (\pm 0.2) \times 10^8$</td>
<td>$3.1 (\pm 0.5) \times 10^8$</td>
<td>$30.5 (\pm 2.7) \times 10^8$</td>
</tr>
</tbody>
</table>

Research on liposomes of different sizes but similar targeting antibody density, for example
Lip (130, 5.8) and Lip (280, 29.3) (Table 2, and 3), demonstrated that the association rate did not increase as the size did, but on the other hand, the dissociation rate was reduced significantly, leading to the increase of the affinity constant as the size of the liposomes increased from 130 nm to 280 nm. Based on these observations, one can conclude that similarly to the increase of the antibody, increase of the size of the liposomes increases the association constant by reducing the dissociation rate.
Figure 15. (a) Sensorgrams of liposomes (280nm) with various target antibody surface densities. To precisely determine the dissociation rate, a prolonged dissociation phase was conducted but was not fully demonstrated. (b) A plot of binding affinity as a function of target antibody surface density. Surface density was interpreted in two ways: antibody per liposome ratio and antibody per millimeter square.
Figure 16. A plot of binding affinity as a function of the liposome size. All three samples were modified with enough target antibody to reach the saturation.

To further study the effect of size, samples of intermediate sizes (183nm) were prepared and modified with sufficient antibody density to reach the saturation point of $1.5 \times 10^8$ antibody/mm$^2$. Then, the determination of the targeting ability was compared for these intermediate sized liposomes to Lip (130, 13.5) and Lip (280, 73.4) as shown in Figure 16. With a similar surface density of the antibody, larger liposomes possessed a higher association rate and a much lower dissociation rate. The most likely explanation for this result is the smaller curvature of larger liposomes, suggesting more surface area available for the contact with the sensor chip. With similar targeting antibody surface density, more targeting antibody molecules on larger liposomes
could bind to the antigen on the surface, causing a more pronounced avidity effect. On the other hand, the Rmax also increased along with the binding affinity, inducing closer packing of larger liposomes on the chip surface.

Thus, with the same ratio between the targeting antibody and the constructive lipid, larger liposomes offer much higher association constant and binding affinity than smaller ones. However, liposomes with smaller size may have other advantages in drug delivery, for example the ability of freely going through certain biological barriers. Thus, the size of the liposomes should be carefully considered according to their particular application.

**Primary antibody**

Results from these experiments indicated that the binding affinity depends on the targeting antibody surface density and liposome size for the secondary antibody model. This raises the question of whether a primary antibody, an actual targeting reagent, will follow similar trends and reach a similar saturation point. Thus, a primary antibody model was developed with anti-mucin1 fiber antibodies in conjunction with a mucin fiber antigen. Mucin fibers are heavily glycosylated proteins (glycoconjugates) produced by epithelial tissues. Mucin-1 (MUC1) is a cell surface associated mucin linked to the apical surface of epithelial cells, thus, providing a good target for epithelial cells. In addition, many kinds of cancer cells overexpress MUC1, offering a potential target for cancer therapy. Anti-MUC1 antibody was previously used as the targeting agent [190]
Similarly to previous experiments, we immobilized MUC1 fragments on the chip using the same mechanism of EDC, resulting in $3 \times 10^{10}$ antigen molecules/mm$^2$, equivalent to 3 ng/mm$^2$. Sensorgram from free antibody analysis indicated a binding constant of anti-MUC1 ($9.0 \pm 1.1$) $\times 10^8$ (M$^{-1}$), which was lower than that for the secondary antibody because of the lower binding affinity of anti-MUC1.
Figure 17. (a) Sensorgrams of liposomes (116nm) with various primary target antibody surface densities. (b) A plot of binding affinity as a function of target antibody surface density.
Another set of the liposomes (116nm in diameter) was generated with varying surface densities of anti-MUC1 antibody (Table 6). Sensorgram for these samples are shown in Figure 17a. The binding affinities seen in Figure 17b were found by fitting these results into the same Langmiur model as previously. Similar saturation trend was observed at approximately $1.5 \times 10^8$ antibody/mm$^2$. Detailed kinetic parameters are shown in Table 6.

In addition, as seen in the free anti-MUC1 sensorgram, the slower dissociation rate again contributed to the increase in binding affinity, indicating the avidity effect relied on the reduced chance of the antibody detaching from the surface, which is similar to the secondary antibody model previously described (Table 6). The trend of Rmax, the theoretical maximum signal for the sensorgram was also similar to that observed in the case of the secondary antibody. By comparing the binding affinity, $\beta$, of the targeted liposomes to that of the free anti-MUC1 antibody, the avidity effect parameter for samples upon reaching the saturation was found to be ~22, consistent with the previously studied secondary antibody model with similarly sized liposomes (130 nm).

These results suggest that the saturation trend, avidity effect, and the saturation point of the targeting antibody surface density were not influenced by the type of targeting antibody or the size of the delivery vehicle, at least in this liposome model. These trends also appeared to be independent on the binding affinity of the targeted antibody used.
5.3.3. Cell binding assay.

Cell binding analysis, which has been used previously for evaluating the affinity of various kinds of targeting antibodies or ligands, was also studied here to verify the results from SPR experiments. Anti-MUC1 was selected as the model antibody. A459 cell line, homo sapiens lung carcinoma, which can produce MUC1 fibers was selected as the cell culture model. Another targeting reagent, Anti-NMDAR1 antibody (anti-NR1), which targets neurons, was also studied. For targeting by anti-NR1, neuroblastoma cells were used as the cell culture model.

For this cell binding assay, sets of rhodamine-labeled liposomes with various the appropriate targeting or control antibody surface densities were prepared. Rhodamine C18 was incorporated into the lipid bilayer of the liposomes during the synthesis. Confluent cells were treated with liposome samples of various dilutions, incubated for 6 hours, and then washed with a buffer to remove the non-specific bound liposomes[178]. In some other studies, the fluorescence from the rhodamine-labeled liposomes was just viewed under microscope to prove the targeting efficacy, but in this study the cells was measured by plate reader to determine the amount of the liposomes bound to cells (Figure 18a &b). As shown in this figure, the amount of liposome binding after incubation was found to increase initially with the increase of the surface density of the targeting antibody, reaching the saturation when the surface density reached the value similar to that found in SPR experiments.
To quantitatively investigate the saturation phenomenon for both targeting antibodies, a method was introduced to quantify the improvement of binding efficiency, by comparing the area under each curve. The targeting index (TI) was defined as the area under the sample curve divided by the area under the control curve produced by the liposomes with non-targeting antibody[192]. This method was originally designed to evaluate the association strength and the washing efficacy in ELISA. The TI as a function of the surface density of the targeting antibody is shown in Figure 18c. Both curves demonstrated saturation behavior with the threshold antibody surface density of approximately $1.6 \times 10^8$ antibody/mm$^2$, in good agreement with the data obtained in the SPR experiments.
Figure 18. Cell binding assay. Plot of fluorescent intensity from immobilized liposomes as the function of dilution times. Anti-MUC1 antibody (a) and Anti-NR1 (b). The comparison of target index of anti-MUC1 and anti-NR1 as the function of antibody surface density (c).
These results showed a good correlation between SPR and cell binding experiments. However, compared to a traditional cell binding assay, SPR approach enjoys several advantages. First, SPR analysis is more focused on the real targeting mechanisms, excluding other factors including the property of the delivery vehicles or cells. For example, polymeric NPs and other widely used nano-sized vehicles cannot be taken up or merged with cells as quickly as liposomes, so their actual targeting efficacy derived from the targeting reagents could be difficult to compare. Second, SPR provides more quantitative measurement and allows one to determine the value of the association constant, increasing the reliability of the experiments. In addition, SPR is a real-time analysis, offering kinetic measurements of both the association rate and dissociation rate. This facilitates the exploration of the in-depth mechanism of binding affinity. The indication in this study that the primary contributor to the avidity effect is the reduced dissociation rate serves as a good example of the advantages of studying the kinetic process. Finally, compared to cell binding analysis, SPR requires a smaller amount of sample for analysis and is a label-free experiment, which could be beneficial in those cases when there is a concern that the properties of the labeled vectors could be different from the properties of the unlabeled vectors.
5.4. Conclusion

The increase of targeting efficacy as the increase of surface density of the targeting antibody derived from the avidity effect reached a plateau at a certain surface density of the targeting antibody. We developed a method to determine the threshold surface density, and this density was found to be consistent regardless of the size and even the type of the targeting antibody. This threshold is believed essential in the design of optimized drug delivery vehicles. Moreover, the developed SPR based methodology can be applied universally to many different delivery systems.
CHAPTER 6

IN VITRO EVALUATION OF THE PROTECTIVE EFFICACY OF SIMULTANEOUSLY DELIVERED ANTIOXIDANT AND GLUCOCORTICOID DRUGS

6.1. Introduction

COPD, the third leading cause of death in the US according to a recent WHO report, includes inflammatory conditions such as chronic bronchitis, emphysema, and airway obstruction, with cigarette smoking being the primary cause [193-195]. Non-smoking COPD is believed to be related to air pollution, including ambient, traffic-related, or indoor air pollution [196, 197].

Currently, this disease has no practical cure, and most of the available treatments are limited to relieving the symptoms and/or slowing its progress [198], and even these, especially the latter, have been found to have limited effectiveness [144, 199].

Similar to most inflammatory diseases, oxidative stress, the central feature of COPD, is considered to be both the initiator and the primary driving force [200]. Given its function, the large surface area and the associated blood supply, the lung is particularly vulnerable to damage from this stress [201]. In the case of COPD, exogenous oxidative stress from cigarette smoke (mainly from the solid phase after combustion) or other air pollutants has been found to be the main factor in the disease’s pathogenesis [202]. This stress leads to a progressive and irreversible loss of
airflow in the lung, an inflammatory response that is resistant, or insensitive, to the glucocorticoid therapy [144, 145], a treatment protocol generally accepted to be effective in the suppression of other inflammatory conditions. Used to treat several inflammatory illnesses [199], glucocorticoids were first widely prescribed in the treatment of the rheumatoid arthritis [203, 204], and then later generalized to other inflammatory situations, for example those affecting the pulmonary and ophthalmic systems [205-207].

Many mechanisms attribute to the anti-inflammatory effect of glucocorticoids, including those responsible for the suppression of pro-inflammatory genes and for the activation of anti-inflammatory ones [208] [209], and the former one was considered as the principle mechanism. Glucocorticoids bind to glucocorticoid receptors (GR) forming an activated GR complex and migrate to the cytoplasm, recruiting the transcriptional co-repressor histone deacetylase-2 (HDAC2) which has the ability is to switch off the many activated inflammatory genes that encode cytokines, chemokines, adhesion molecules, and inflammatory enzymes [27, 209].

In general, HDAC is able is to remove acetyl moieties from histones, resulting in the condensation of the chromatin structure around the histone cores preventing their transcription [210], meaning it can silences the genes. HDAC2 is one of the 18 isoforms in the HDAC family that has been hypothesized to play a central role in the transcription of genes associated with NF-kappaB and other pro-inflammatory factors [211-213]. On the other hand, histone acetylation
by histone acetyltransferases (HATs) disrupts the attractive electrostatic interaction between the DNA and the histone, resulting in the unwrapping of the DNA. The balance between HDAC2 and HAT is crucial for pro-inflammatory gene expression. Previous studies have shown that the high oxidative stress inhibits both the activity and the production of HDAC2 and in COPD patients, thus enhancing gene expression of pro-inflammatory factors [214, 215] (Figure 19).

Figure 19. Glucocorticoid suppression of activated inflammatory genes. Inflammatory stimulus activates the inhibitor of nuclear factor kappa B kinase, which therefore activates NF-kappa B. A dimer of p50 and p65 NF-kappa B proteins translocates to the nucleus and binds to specific kappa B recognition sites on the promoter regions of inflammatory genes and to co-activators such as the cyclic AMP response element binding protein (CBP). The co-activator causes acetylation of core histones, activating gene expression of inflammatory proteins. Activated glucocorticoid receptors (GRs)
bind to coactivators in the nucleus to inhibit histone acetyltransferase (HAT) activity directly. GRs also recruit histone deacetylase 2 (HDAC2), leading to the suppression of the activated inflammatory genes.

The idea of restoring the production and activity of HDAC2 by introducing antioxidants have been proven by using polyphenols [200], Nrf2 activator [216], and enzyme mimetics. Thus, we hypothesize the co-delivery of glucocorticoids with antioxidants can restore the anti-inflammatory efficacy of glucocorticoids. In addition, since in the case of COPD, oxidative stress is not only the primary driving force of the inflammatory response but also one of the initial factors, antioxidants are also considered as potential therapeutic agents for COPD itself. However, limited studies have focused on the co-delivery of both antioxidants and glucocorticoids to create a synergistic effect [217, 218]. To address this need, the study reported here used nano-sized liposomes to deliver both antioxidants and glucocorticoids to the lungs to improve the treatment of COPD.

Recently, such nano-carrier systems have received increasing attention in pulmonary drug delivery especially for local lung diseases. The application of nano-carriers in lung offers several advantages including 1) the potential to achieve relatively uniform distribution of a drug dose among the alveoli; 2) enhanced solubility of the DEX than its aqueous solubility (glucocorticoids);
3) the sustained release of a drug which consequently reduces the dosing frequency; 4) the suitability for delivery of macromolecules (SOD enzyme); 5) a decreased incidence of side effects; 6) improved patient compliance; 7) the potential of drug internalization by cells; and 8) increased stability. [219].

Liposomes, colloidal dispersions with self-assembling structures due to the intrinsic interfacial chemistry of phospholipids, enjoy additional advantages for pulmonary administration over other nano-sized carriers and polymeric nanoparticles. Most importantly, liposomes, which are able to encapsulate hydrophilic, hydrophobic, or amphiphilic substances in either the core or the bilayer membrane without molecular modification, have a high drug loading capacity [220].

Like most nano-medicines for pulmonary delivery, in earlier studies, liposomes were designed to be administrated by nebulizer [221]. However, because of their high surface energy, the disturbance during nebulization can cause agglomeration in the colloidal dispersions. Moreover, the chemical stability of colloidal dispersions is another problem due to the hydrolysis and degradation of both the liposomes and drugs [222]. Therefore, in recent studies, liposomes were designed to be freeze-dried for storage, and later the micron-sized powder of the aggregation of nano-sized particles would be administrated by DPIs [223, 224].
6.2. Materials and Methods

6.2.1. Materials

The lipopolysaccharide (LPS) was purchased from ENZO Life Science as were an A549 Homo sapiens lung carcinoma cell and RAW 264.7, while a mouse leukaemic monocyte macrophage cell line was purchased from ATCC. Dulbecco’s Modified Eagle Medium (DMEM), Hank’s Balanced Salt Solution (HBSS), Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Trypsin EDTA 1X in HBSS, and Antibiotic/Antimycotic (anti/anti) solution were purchased from Cellgro Mediatech, Inc. An E.Z.N.A Total RNA kit was purchased from Omega Bio-Tek, Inc., and a PerfeCTa SYBR Green FastMix PCR Kit and a qScript cDNA SuperMiX were purchased from Quanta Biosciences, Inc. Primers for the targeting and housekeeping genes were purchased from Integrated DNA Technologies, Inc. DNAse, RNAse and Protease free water were purchased from Acros Organics. Human interleukin 8 (IL-8) ELISA MAX Deluxe was purchased from BioLegend, Inc., and MTT Cell Proliferation Assay from ATCC. All other chemicals were purchased from Sigma-Aldrich and used as received unless otherwise specified.

6.2.2. Cell Culture Models

Cell cultures of both A549 and RAW 264.7 were passaged in non-surface treatment T75 flasks with filter caps. These cultures were subsequently maintained using DMEM with 10% FBS (heat inactivated) and a 1% anti/anti solution. This mixed medium was further filtered through a 0.2 μm
vacuum filtration systems. When the amplification of the cells reached approximately 90% confluence, they were collected for the next passage. After proliferation, cells were seeded onto 24 or 48 well-plates at a concentration of $10^4-10^5$ cells per mL one day before the experiments. A 0.25% trypsin solution was used to remove the cells from the surface of the flasks between generations. All cell lines were maintained in a continuous culture at 37 °C and 5% CO$_2$ in an incubator.

For the A549 and RAW 264.7 co-culture, cells were collected and mixed at a 5:1(A549: RAW 264.7) ratio and later seeded on well-plates.

### 6.2.3. Cell Treatment

LPS was dissolved in the mixed DMEM medium, the resulting concentrations selected to induce a significant but submaximal release of IL-8 and other inflammatory signals. Initial experiments were conducted to obtain a concentration response curve of effect of the LPS (1-1000 ng/mL) on the release of IL-8. According to previous research, A549, a type of carcinoma cell, exhibits increased tolerance for abnormal situations, and thus, the effective challenging concentration of LPS should be higher for it than for other primary cells [225]. H$_2$O$_2$ was used in the subsequent experiments to mimic the oxidative stress in the lungs of COPD patients. Similar to the LPS, an initial experiment was also conducted to determine the appropriate H$_2$O$_2$ concentration for inducing enough oxidative stress to introduce glucocorticoid repression in conjunction with the LPS challenge but only a limited inflammatory response [226].
Protective drugs, glucocorticoids and antioxidants, were also dissolved in the DMEM medium mixture. A 20 μg/mL DEX stock solution was prepared by dissolving 1mg DEX in 1 mL absolute ethanol and diluting it to create a 50 mL medium mixture. For the drug treatment experiment, the cells cultured in the well plates were incubated with drugs or liposome-loaded drugs one hour earlier than the LPS challenge or the combination of LPS and hydrogen peroxide.

**Determination of the ROS Production**

The ROS in cells was determined using 6-carboxy-2,7-dichlorodihydro -fluoresceindiacetate (DCFH-DA). After entering the cells, DCHF-DA, an ester that is freely permeable across the cell membrane, loses the diacetate group by esterase in the cells, becoming DCFH, which is not permeable across the membrane. Here DCFH functioned as a substrate for oxidation by ROS, producing a highly fluorescent intracellular product, DCF. For the ROS detection assay, the cells were seeded in 96 well plates. After being treated with LPS and other challenges with or without protection from drugs, the cells were washed twice with a medium and then incubated with DCFH-DA (10 μM) for 30 min at 37°C in the cell culture incubator. After a washing with PBS, the fluorescence was measured using a fluorescence microplate reader (excitation λ 495nm and emission λ 520nm) [227]. Hydrogen peroxide was included as the positive control.

**6.2.4. Released IL-8 Analysis by ELISA**

IL-8, a pro-inflammatory mediator, was used as the index of the inflammatory level. In this
study, the amount of IL-8 released was measured using a commercially available enzyme-linked immunoassay assay (ELISA). The supernatants of the cell culture experiments were collected, diluted, and assayed according to the manufacturer’s protocol. The optical density (OD) at 450nm was measured using a microplate reader. The relationship between the OD at 450nm and the IL-8 concentration was obtained by adding the standard samples from the serial dilution of the LPS solution with a known concentration to the assay. The final concentrations of each sample were calculated as the means of the results from the sample dilution yielding ODs in the linear region of the calibration curves.

6.2.5. Gene Expression Measured Through Quantitative PCR

To further investigate the protective effect of the glucocorticoids and the ability of antioxidants to restore their function, quantitative PCR was used to analyze the detailed alterations in the gene expression, beginning with the extraction of the RNA from the in vitro experiments and its purification. This extraction of all RNA from the cell lysates was achieved by using the EZNA total RNA kit according to the manufacturer’s protocol. After the trypsinitization and homogenization of the cells in a lysis buffer, the samples were translated to a HiBing RNA column with a selective membrane, which uniquely retains the RNA while washing buffers remove the proteins. The RNA was subsequently eluted from the column by adding DEPC-treated water. The amount of RNA was measured using the absorbance of its solution at 260nm, and the ratio of absorbance at 260nm over
280nm was measured and found to be in the range of 1.98-2, indicating a pure RNA solution.

After the RNA extraction, a reverse transcription reaction was conducted using qScript XLT cDNA SuperMix. Based on the protocol, test tubes containing a mixture of the extracted RNA and superMix were incubated at 25 °C for 5 minutes, followed by 60 minutes of incubation at 42 °C, and finally, the test tubes were held at 85 °C for another 5 minutes in a Rotor-Gene 3000 Thermal Cycler (Corbet Research, Quiagen Inc.).

Quantitative PCR analysis was conducted using a PerfeCTa SYBR Green FastMix PCR Kit based on the manufacturer’s instructions, also in the Rotor-Gene 3000 Thermal Cycler. The cDNA, SYBR Green FastMix PCR Kit and the primer mixtures, their composition described below, were initially denatured at 95°C for 15 min. Amplification was then conducted using the following thermal cycling: 94°C for 20 sec, 60°C for 20 sec and 72°C for 15 sec, all with 45 cycles. Finally, melt curves were obtained by increasing the temperature from 25°C to 85°C.

Primers were designed as follows (annealing temperature and product size appear in parentheses): IL-8 sense 5' - ACT GAG AGT GAT TGA GAG TGG AC -3' and antisense 5' - AAC CCT CTG CAC CCA GTT TTC -3'; NF kappa B sense 5' - ATG TAT GTG AAG GCC CAT CC -3' and antisense 5' - TTG CTG GTC CCA CAT AGT TG -3'; HDAC2 sense 5' - CCC TGA ATT TGA CAG TCT CAC C -3' and antisense 5' - CAC AAT AAA ACT TGC CCA GAA AAA -3'; the housekeeping gene GAPDH sense 5' - TCA AGG CTG AGA ACG GGA AG -3' and antisense 5' -
ATG GTG GTG AAG ACG CCA G -3' [228]. The threshold level was set sufficiently above the background noise. The CT value for each sample fluorescence curve was determined at the point where the fluorescence curve crossed the threshold line. For this experiment, 0.02 was selected as the threshold. In a preliminary experiment, the efficiency of the targeting and housekeeping gene primers was calculated from the CT values of the serially diluted cDNA solution with known concentrations, with 100% efficiency indicating a doubling of the amount of the amplified sequence in each PCR cycle.

Quantitative analysis of gene expression was calculated based on the Ct from a targeting gene (A) and a housekeeping gene (B) for the two cell types, untreated cells and sample cells. Thus, the relative gene expression was represented by this formula:

\[ \frac{N_{\text{sample}}}{N_{\text{untreated}}} = 2^{-\Delta\Delta\text{C}_T} \]

\[ -\Delta\Delta\text{C}_T = (\text{C}_{T,\text{untreated},A} - \text{C}_{T,\text{untreated},B}) - (\text{C}_{T,\text{sample},A} - \text{C}_{T,\text{sample},B}) \]

where \( N_{\text{sample}} \) and \( N_{\text{untreated}} \) represent the number of targeting genes copies in the untreated and samples cells, respectively, and \( C_{T,\text{untreated},A}, C_{T,\text{untreated},B}, C_{T,\text{sample},A} \) and \( C_{T,\text{sample},B} \), the average CT values calculated from three PCR replicates for both the targeting gene (A) and the housekeeping gene (B). This approach allows the gene amount from the samples with various cell amounts to be normalized by the housekeeping gene.
6.2.6. Statistical Analysis

The values of the protein amount obtained from ELISA, the ROS amount obtained from the DCFDA assay, and the gene expression determined from quantitative PCR were compared using the t-test. The number of replicates was n>3.

6.3. Results and Discussion

6.3.1. LPS Challenge on A549 Cells

LPS, also known as endotoxin, is a large molecule composed of a covalently bonded lipid and polysaccharide found in the outer membrane of gram-negative bacteria. It has been frequently applied to induce inflammatory responses in many cell types in in vitro experiments [229]. In this study, LPS was used as the main stimulator of inflammatory response.

IL-8 is one of the chemokines produced by many types of cells including epithelial cells, the main function of which is to attract granulocytes. Thus, IL-8 has been applied to evaluate the level of inflammatory reaction in both in vitro and in vivo experiments. Here, commercially available direct sandwich ELISA conducted in 96 well plates was used to determine the inflammatory level after LPS activation.

The concentration of the released IL-8 in the medium from A549 cells was plotted against the
various concentrations of the LPS used at 12 hrs time point in Figure 20A. The results suggested that the released concentration of IL-8 was positively related to the concentration of LPS used, reaching a maximum at approximately 1000 μg/mL. Further increased LPS concentration induced obvious cell apoptosis (not shown in the figure). Here 100μg/mL and 10 μg/mL (853±120 and 720 ±66 pg/mL) were selected for further investigation because the released IL-8 was significantly higher than the baseline (312±34 pg/mL, P<0.001) but did not reach the peak secretion. In comparison, this concentration was much higher than that used in most studies using primer cells (500 ~ 10 ng/mL) [230], but was consistent with those using A549 cells [226].
Figure 20. DEX reduces the inflammatory response in A495 cells stimulated by the LPS challenge. (a) Concentration-dependent effect of the challenged LPS on IL-8 release in A549 cells over a period of 12 hrs. (b) The concentration-dependent effect of dexamethasone on LPS challenged cells in 12 hrs. (c) ROS production from the LPS challenged A549 cells.
With the appropriate concentration of LPS (10 μg/mL) as the challenge, an initial DEX protective experiment was conducted. Previous studies found that the presence of LPS leads to an elevation of the ROS level in A549 cells, perhaps inducing glucocorticoid resistance [231]. However, as shown in Figure 20B, the IL-8 production was effectively suppressed to the baseline level by even a low concentration of DEX (0.001 uM, 351±30 pg/mL in comparison to a positive control 873±100 pg/mL, P<0.001), which is the lowest practical concentration used in previous in vitro experiments [226]. Thus, no obvious glucocorticoid resistance was observed from the LPS challenge alone. The results of the DCFH-DA assay analyzing the ROS generation in A549 with LPS can be seen in Figure 20C. Unlike as found in several previous studies, the serial concentrations of LPS did not induce any relative change in the fluorescent intensity from the DCF, and the fluorescent intensity of the cells treated with the highest concentration of LPS (100 μg/mL) was not significantly higher than the negative control. In addition, the combination of LPS and DEX also did not result in an increase in fluorescence. Here 100 μM H$_2$O$_2$ was included as positive control based on the standard protocol.

This inconsistency of the ROS production may be a result of the incubation environment and the time of cell collection. Although in the process of inflammatory response, both epithelial cells and macrophages can produce ROS, the alveolar macrophages have been found to produce most of
the increased amount of ROS, including oxygen free radicals and hydrogen peroxide [232, 233]

6.3.2. Co-culture of A549 and Macrophages

Activated macrophages produce oxygen free radicals through the activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, which is a complex enzyme system present in phagocytes.
Figure 21. Addition of RAW 264.7 macrophages into the in vitro model. (a) Concentration dependent effect of LPS induced ROS production in RAW 264.7 macrophages (0.001 μM of DEX). (b) LPS treated A549 and RAW 264.7 cell co-culture. A549 and RAW 264.7 were seeded on wells at a ratio of 5:1. Two time points were included.

We used a co-culture of A549 and RAW 264.7 macrophages, a combination developed to mimic pulmonary tissue composition in previous studies [234]. A preliminary test was conducted to evaluate the ROS production via a DCF assay. Figure 21A showed a significant increase in the ROS production after the treatment of LPS (9458 ±1520 versus 2045 ± 367 of negative with P<0.01). In addition, as the LPS concentration increased, the ROS production also increased (9458 ±1520 from 10 μM versus 4239 ±567 from 0.1 μM). This proved that, the AW 264.7 macrophages
can produce ROS by the challenge of LPS.

However, as shown in this figure, the addition of DEX (0.1 uM) effectively reduced the production of ROS (9458 ±1520 versus 3624 ± 1235 P<0.01), and the reduced ROS concentration was not significantly different from the baseline control of non-treated cells, indicating that the ROS produced from RAW 263.7 may not be enough to generate glucocorticoid resistance.

To investigate that if the ROS produced from RAW 264.7 was enough to generate glucocorticoid, a co-culture system with both RAW 264.7 and A549 cells was used. Similar to previous studies, cells were amplified in flasks separately, and subsequently, the two harvested cell suspensions were mixed at a 5:1 (A549: RAW 264.7) ratio, before seeded on well-plates. As illustrated in Figure 21B, the co-culture system was more sensitive to stimulation from LPS, a higher concentration of IL-8 being observed after an incubation period of 12 hours, because macrophages play an essential role in the amplification of inflammatory responses by producing many types of cytokines, including IL-8. However, as shown in the figure, the addition of DEX also results in the reduce of the IL-8 levels, especially for short-term incubation. More specifically, even the lowest practical DEX concentration (0.01 μM) can significantly reduce the amount of IL-8 released at both the 12 and 6 hour time point.

Based on the results from both the DCF-DA assay and the IL-8 ELISA, endogenous ROS from the co-culture of A549 and RAW 264.7 was found to be not enough to induce obvious
glucocorticoid resistance, and, therefore, exogenous ROS appears to be necessary to establish a model to evaluate the synergistic effect of both antioxidant and glucocorticoid drugs.

6.3.3. Exogenous ROS-Induced Glucocorticoid Resistance

Hydrogen peroxide, one kind of ROS, plays a pivotal role in inflammatory response. Many enzymes and reactions relate to hydrogen peroxide, for example glutathione peroxidase, superoxide dismutase, catalase, and Fenton chemistry. For this reason, hydrogen peroxide has been considered as a potential reagent for introducing oxidative stress in *in vitro* experiments. Previous studies have found that hydrogen peroxide at an appropriate concentration alone does not increase the inflammatory response but is enough to generate glucocorticoid resistance. Here the concentration dependent glucocorticoid resistance from hydrogen peroxide was analyzed by determining the concentration of the IL-8 released in Figure 22. As the figure shown, the low concentration of hydrogen peroxide (10 μM) did not induce an inflammatory response or block the function of DEX. On the other hand, a high concentration of hydrogen peroxide (1000 μM) induced a significant increase in the release of IL-8 (1011±43 pg/mL), and an even higher concentration caused significant cell death (not shown in the figure). For this study, hydrogen peroxide with 100 μM in concentration, which effectively impacted the function of the DEX but did not increase the inflammatory response, was selected for further investigation.
Figure 22. Hydrogen peroxide was applied as exogenous oxidative stress. The concentration dependent glucocorticoid resistance effect on A549 cells.

6.3.4. Antioxidants Restoration of the Function of Dexamethasone.

Many antioxidants have been claimed effective to restore the function of glucocorticoids, including polyphenols, spin traps, and cysteine alternatives, indicating their potential as improved therapeutic agents for glucocorticoid-resistant COPD.
Figure 23. Effect of SOD and SOD mimetic on LPS and hydrogen peroxide stimulated A549 cells. Cells were first incubated with DEX and SOD/SOD mimetic and later challenged with LPS and hydrogen.

This study used SOD and SOD mimetic as antioxidants to restore the function of glucocorticoids. To investigate their performance, it used the COPD model with a combination of LPS and hydrogen peroxide. As shown in Figure 23, DEX alone did not recover the concentration of released IL-8, but the addition of SOD or SOD mimetic at a high concentration (1KU/mL) restored its level to the baseline. As the decrease of the activity of SOD and SOD mimetic used, their function also diminished, and the level of the IL-8 released increased. In addition, as clearly demonstrated, with the same activity, the SOD mimetic exhibited a much better protective effect than SOD, for example at 1 kU/mL 307.5 ±43.8 pg/mL versus 436.4 ±31.5 pg/mL with p<0.01, and
at 100 u/mL 311.3 ±30.8 pg/mg versus 658.6 ±12.2 pg/mL with p<0.001.

To explore detailed response of the cell by the challenge of LPS and hydrogen peroxide and the treatment of the DEX and antioxidants, the change of gene expression of several biomarkers was determined via quantitative PCR. In the mechanism of glucocorticoid resistance, oxidative stress reduces the expression of HDAC2, thus blocking the anti-inflammatory efficiency of DEX, and LPS stimulates inflammatory response via NF-kappa B. Therefore, besides IL-8, the gene expression of HDAC2 and NF-kappa B were analyzed (Figure 24). As Figure 24A shown, the expression of the IL-8 gene was quite consistent with its released concentration determined by ELISA. Similarly, the single drug of DEX did not restore the IL-8 gene expression to its normal level (3.51±0.23 folds of the IL-8 baseline), while the addition of SOD and SOD mimetic significantly reduced the IL-8 gene expression (1.50±0.06 and 1.95±0.11 folds of 1 kU/mL SOD and SOD mimetic respectively). Similar to the IL-8 ELISA result, the protective effect of SOD/SOD mimetic at lower concentrations was diminished, especially for the SOD enzyme.

NF-kappa B can be stimulated by such exogenous stimulators, such as LPS, TNF-alpha, and environmental ROS to facilitate the transcription of inflammatory genes. With stimulation by LPS, its gene expression was raised to more than 2 folds, as seen in Figure 24B. The introduction of hydrogen peroxide and the addition of DEX did not change the expression of NF-kappa B. The addition of hydrogen peroxide and antioxidants did not significantly change its gene expression.
level, indicating that the addition of hydrogen peroxide did not lead to the increase of inflammatory response, but just blocked the function of glucocorticoids.

In addition, results from the gene expression of HDAC2 demonstrated that even challenged by a high concentration of LPS, the production of HDAC remained at the normal baseline level (0.90±0.13 fold) as seen in Figure 24C. However, the introduction of hydrogen peroxide significantly reduced its expression level (0.54±0.04 fold), and the addition of DEX did not recover this decrease. Once a high concentration of antioxidants was introduced, especially SOD mimetic, HDAC2 expression was restored to normal level (0.89±0.10). The gene expression of HDAC2 also showed that the effect of SOD mimetic was much better than that of the SOD enzyme.

In conclusion, these results further support that oxidative stress is the primary cause of the reduction of HDAC2 expression and the subsequent glucocorticoid resistance. In addition, the antioxidant used here effectively restored the level of HDAC2. In contrast, most previous studies just focused on the recovery of DHAC2 production and its activity. However, this study not only suggested that a high concentration of potential antioxidant (SOD and SOD mimetic) can recover the HDAC2 gene expression but also demonstrated that this recovery of the function of HDAC2 restored the anti-inflammatory effect of DEX. This result confirms the hypothesis of the co-delivery of antioxidant and glucocorticoid drugs.
Moreover, it is known that SOD and SOD mimetic exhibit similar active units per mg (approximately 6000 U/mg), while the cytotoxicity of SOD mimetic is much lower than the SOD enzyme. In addition, SOD mimetic offers other advantages over the SOD enzyme, including a lower immunological response and more stability. Thus, this study used the SOD mimetic for further co-delivery experiments.
Figure 24. The relative gene expression of several essential biomarkers in inflammatory response. (A) IL-8, (B) NF-kappa B, and (C) HDAC2. Here, a non-treated sample was included as the basal negative control. (SOD/SOD mimetic: +++=1000 U/mL, +=100 U/mL)

6.3.5. Liposomes Loaded Antioxidants and Glucocorticoids

The approach to load both DEX and antioxidant onto liposomes simultaneously was illustrated in the Aim 1. In the preparation, the ratio between those two drugs was adjusted based on the results obtained in Figure 23(1KU of SOD mimetic for 0.1 uM of DEX).

The anti-inflammatory effect of the drug-loaded liposomes was analyzed using the same inflammatory model of A549 cells challenged by both LPS and hydrogen peroxide. Both the
concentrations of the IL-8 released (421±78 pg/mL) and the IL-8 gene expressions (1.12±0.12 folds) of the drug-loaded liposomes were similar to the free drug mix (Figure 25a &b). The drug-loaded liposomes exhibited a much better effect on the recovery of the HDAC2 gene expression compared with the free drugs mixture (0.87±0.06 versus 1.13±0.08 with P<0.01). Similar to the free drug mix, drug-loaded liposomes did not influence the gene expression of NF-kappa B. Plain liposomes, which were included as a control to eliminate the influence from the nano-sized vehicle.
Figure 25. Comparison of free drug and liposome-loaded drugs. The plain liposomes were included as a control. (A) IL-8 released from A549 cells after LPS and H2O2 stimulation. IL-8, DHAC2, and NF-kappaB gene expression are also illustrated (B, C, and D).

The improvement of the protective effect of the drug-loaded liposomes was not demonstrated in this static in vitro model. There was no significant improvement of drug-loaded liposomes compared with free drug mixture. In a real situation, the clearance in the pulmonary system is strong, and liposomes will facilitate the cellular uptaken of drugs.

To investigate the advantage of the liposome-loaded drugs, the model was modified by a pre-incubation with drugs for 2 hrs and followed by several washing steps using HBSS buffer to
remove the drugs not internalized by cells. Then, the cells were challenged by LPS and hydrogen peroxide. The level of released IL-8 was analyzed by IL-8 ELISA (Figure 26). As can be seen, the anti-inflammatory effect of the free drugs was compromised compared with the previous model (from ~90% recovery of IL-8 to ~50%) Meanwhile, the drugs loaded on the liposomes exhibited a much better effect in reducing the amount of released IL-8 compared with the free drugs (630±45 pg/mL versus 302±69 pg/mL with P< 0.01). This result indicates that as a delivery vehicle, liposomes improved the cell-availability and increased the amount of internalized drugs in short term.

Figure 26. The IL-8 released from cells at 8 hours after the LPS and H₂O₂ challenge.

Drugs or liposome-loaded drugs were incubated with cells for 2 hrs and washed before the challenge.
Drug-loaded liposomes modified with the targeting antibody were also included in the assay of the amount of released IL-8. The results from Aim 2 suggested that the introduction of the targeting antibody (anti-muc1 antibody) should lead to the specific binding of the liposomes to epithelial cells, including A549, thus further facilitating the internalization of the liposomes. However, as shown in Figure 26, in this unique static in vitro model, the improvement of antibody-modified liposomes was not apparent.

6.4. Conclusion

With the model consisting of the A549 cells challenged with LPS and hydrogen peroxide, the antioxidant, SOD mimetic not only restored the expression level of DHAC2, just as most studies have found, but also removed the glucocorticoid resistance. Liposomes loaded with both the SOD mimetic and DEX can effectively reduce the inflammatory level, returning it back to normal. Furthermore, liposomes increased the bio-availability of the drugs and increased the possibility of internalization by the cells, further improving the protective effect. These results concerning the implementation of a co-delivery liposome delivery system open new possibilities in the treatment of COPD.
CHAPTER 7

CONCLUSIONS AND FUTURE RECOMMENDATIONS

7.1. Conclusion

Overall, the design of the co-delivery of glucocorticoid and antioxidants in one nano-sized targeted delivery system to treat inflammation resulting from COPD was successful in our \textit{in vitro} experiments, and this study addressed its three goals for the development of an effective drug delivery system. First, targeted drug delivery devices carrying both antioxidant (SOD/SOD mimetic) and glucocorticoid (DEX) drugs were prepared and characterized. Second, the effect of the density of the targeting antibody on the affinity of nanoscale drug carriers was investigated, and the optimal antibody density was determined. Finally, the synergistic effect between the glucocorticoid and antioxidant drugs was evaluated using the A549 cell culture model.

The first goal was based on previous observations in the nano-carrier literature. First, according to previous experiments, several kinds of nano-carriers with drugs were prepared and characterized. Their size and zeta-potential were analyzed, the results showing that they were all in the appropriate size range of nanometer, and with an absolute value of zeta-potential to ensure their stability as colloidal suspensions.

Second, relatively high loading capacities of all three kinds of therapeutic molecules
(dexamethasone, SOD, and SOD mimetic) were achieved with liposomes compared to alternative nano-carriers. To deliver the same dose of a therapeutic molecule required, a relatively lower dose of nano-carrier if the loading capacity was high, reducing the toxicity from the carrier itself. In addition, a high loading capacity offered the space needed for the manipulation required to load both drugs simultaneously. For this manipulation, the ideal molecular ratio between the glucocorticoid and antioxidant, would guide the preparation of the nano-carriers. However, the liposomes used here exhibited the capability for loading all three kinds of therapeutic molecules, suggesting the potential of the development of liposomes loading both glucocorticoids and antioxidant.

The stability of the nano-carrier candidates was analyzed through the process of lyophilization and the long-term storage. Polysaccharides, cryoprotectants, were added to prevent the aggregation of nano-carriers during the lyophilization, with the results indicating that liposomes demonstrated much less aggregation. Size is one of the most crucial advantages of nano-carriers as drug delivery vehicles. Meanwhile, the liposomes with the cryoprotector retained most of the original SOD activity, indicating that structure remained intact through lyophilization and reconstitution. Subsequently, an accelerated aging experiment was conducted, indicating the enhanced ability of liposomes to retain their SOD activity during the long-term room temperature storage compared with other candidates. Thus, based on the loading capacity and the stability, liposomes were
selected for preparing the co-delivery system. Lyophilization was required because of the instability of the colloidal suspension and their application in the dry powder inhaler (DPI). Because of the degradation of drugs and liposomes in the aqueous phase and the inconvenience of a nebulizer, currently DPI is the preferred device of application.

The release profile of the glucocorticoid was analyzed using albumin as a transport vehicle for the drug, mimicking the physiological situation of the body. Unlike for water soluble molecules, the transportation of hydrophobic drugs, for example hormones, is facilitated by proteins such as albumin. Unlike free drugs, dexamethasone loaded in liposomes achieved a sustained release over the initial 4-5 days, reducing the frequency of drug administration.

The pilot co-delivery liposome system was prepared to verify the feasibility of loading two kinds of therapeutic molecules by separate mechanisms. Both SOD/DEX and SOD mimetic/DEX liposomes were prepared pursuing the highest loading capacity. There was neither significant decrease of final loading capacity nor the change of physical characteristics, indicating the liposome system was a suitable delivery vehicle in this study and ready for further investigation.

The optimized surface density of the targeting antibody was determined through the modified surface plasmon resonance (SPR) analysis. First, unlike the normal application of SPR, the antigen molecules were immobilized on a sensor chip, while the targeting antibody was running in the channel along with the running buffer. A sensor chip with a high surface density of antigen
molecules was selected to mimic the actual situation in the body.

Then, a secondary antibody was applied as a polite model in the investigation of the influence of antibody amount and of the particle size. Avidity effect was known to raise the binding affinity (targeting efficacy) of liposomes with multiple targeting antibodies, and the targeting efficacy increased with an increase in the amount of targeting antibody. However, this trend saturates at a certain surface density of the targeting antibody. This saturation point was demonstrated and determined by the SPR experiments. Further experiments showed that the saturation point relied on the certain surface density of the targeting antibody, regardless of the size of liposomes.

With the experience gathered from the secondary antibody, the actual primary antibody, anti-MUC1, was analyzed using a sensor chip with the immobilized fragments of mucin 1. The antibody surface density related binding affinity demonstrated the saturation point of the targeting efficacy, which was found to be similar the value found in the secondary antibody experiments. This result indicated that the saturation point might be an essential property which depends only on the nano-carrier itself. The saturation point was crucial in the optimization of the targeting efficacy, maximizing the targeting efficacy from a limited amount of antibody.

The conventional cell binding method was also operated in this study to determine the targeting efficacy, which verified the surface density of the saturation point. However, SPR demonstrated several advantages compared with the conventional method. First, a detailed
investigation of the kinetic parameters revealed that the increased binding affinity was primarily derived from the decreased dissociation rate. Second, the reusability of the chip provided a much easier and faster method for determining the targeting efficacy. In addition, a smaller sample amount was required for the analysis. Last, but most important, SPR as a more quantitative method, is optimal for the comparison between samples.

*In vitro* models were able to demonstrate the synergistic effect derived from the co-delivery of glucocorticoid and antioxidant. First, the model was developed not only by stimulating the A549 cell with lipopolysaccharide (LPS) as is done most for *in vitro* inflammatory model but also by including hydrogen peroxide as an exogenous ROS. That was because that preliminary studies showed that the endogenous ROS was not capable of generating the glucocorticoids resistance *in vitro* even when A549 cells were co-cultured with macrophage cells.

The measurement of the concentration of the released Interleukin 8 as the index of the inflammatory level was determined by the commercially available ELISA kit. As indicated by the concentration of IL-8, the ability of DEX to reduce its level of IL-8 when stimulated by LPS was hindered by the addition of hydrogen peroxide, a condition addressed by the addition of antioxidants, SOD or SOD mimetic.

In addition, the gene expression of histone deacetylase-2 (HDAC2) was found to be suppressed by increased oxidative stress, considered as the primary cause of glucocorticoid
resistance. The addition of antioxidants restored the level of HDAC2 gene expression, while the gene expression of NF-kappa B remained unchanged, indicating that the stimulation of the inflammatory response was uniquely from LPS and that the addition of exogenous ROS did not increase the stimulation but only compromised the anti-inflammatory function of the glucocorticoids.

The liposomes loaded with both glucocorticoid and antioxidant were also included in the analysis, which processed the optimized ratio between these two drugs. A similar protective effect was discovered compared to the free drug mixture in this static cell culture model. To demonstrate the advantage of liposomes, a washing step was introduced into this model to remove the drugs neither binding to nor uptaken by the cells. Results from both the release of IL-8 concentration and the gene expression of several factors suggested an enhanced protective effect from liposomes compared to the free drugs mixture, revealing an increase in the cell availability of those drugs loaded by liposomes.

In addition, the results demonstrated that SOD mimetic performed much better in this in vitro model compared to the actual SOD with the same amount of the active unit. In consideration of other advantages of the SOD mimetic, for example its higher stability, smaller molecular weight, and less immunogenicity, this metalloporphyrin, was selected over SOD. This study also included liposomes decorated with the targeting antibody, anti-muc1.
7.2. Future Directions

While this work focused on the *in vitro* protective efficacy reported here, there is potential for application in the treatment of COPD. However, much work remains to be done.

One study could involve detailed analysis of the characteristics of the liposomes-loaded drugs. First, detailed size-distributions and shapes of liposomes could be determined by such techniques as atomic force microscopy (AFM) or scanning electron microscope (SEM). That is important, because the DLS applied in this study is claimed not ideal for analyzing their sizes. Furthermore, their composition could be analyzed including the drug molecules and each lipid components, and their distribution in the liposomes. This property is crucial for predicting the body’s response to this therapeutic system, including its degradation and bio-distribution.

The ultimate goal for this liposome is to be delivered into the respiratory system by DPI. The property of the freeze-dried powder is important for this application as it contains not only aggregated liposome particles but also covered by polysaccharides, cryoprotectors, meaning the size is larger than that of liposome. This increased size would need to be determined because it is crucial to the nano-carriers’ deposition location in the respiratory system. Other drying techniques could also be applied if it is necessary.

A rat model could also be used to analyze the distribution and targeting efficacy of liposomes. First, a nasal drop administration could be applied to evaluate the targeting efficacy of samples with
and without the targeting antibody. Subsequently, dry powder could be administrated to
demonstrate the bio-distribution of this liposomes system.

To explore the ability of this liposomes system to effectively treat COPD, our collaborators at
the Medical University of South Carolina will develop a rat model with this condition. with rats
exposed to sidestream cigarette smoke in a cigarette smoke chamber. Then, a detailed comparison
of the rats with liposomes administration and the control group will be conducted, primarily
focused on the harvested lung tissue. If the rat study is successful and indicates an improvements in
the anti-inflammatory functions compared with traditional DEX administration, then a small scale
human trial will be conducted.

Future work could also include the generalized application of this SPR technique. Given its
advantages compared with the conventional methods, this method may be applicable for any
targeting molecule. In addition, other types of nano-sized carriers besides liposomes could also be
analyzed using this method.
APPENDICES

APPENDIX A: Abbreviations

BSA- Bovine serum albumin

COPD- Chronic obstructive pulmonary disease

DCF- 2',7'-dichlorodihydrofluorescein diacetate

DEX- Dexamethasone

DLS- Dynamic light scattering

DMEM- Dulbecco’s Modified Eagles Medium

DPI- Dry powder inhaler

ELISA- Enzyme-linked immunosorbent assay

FBS- Fetal bovine serum

GR- Glucocorticoid receptor

HAT- Histone acetyltransferases

HDAC2- Histone deacetylases 2

IL-8- Interleukin 8
LPS- Lipopolysaccharide

MDI- Metered dose inhaler

MUC1- Mucin 1

NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells

NP- Nanoparticle

PCR- Polymerase chain reaction

PLA- Polylactic acid

PLGA- Poly(lactic-co-glycolic acid)

PVA- Polyvinyl alcohol

ROS- Reactive oxygen species

SOD- Superoxide dismutase

SPR- Surface plasmon resonance
APPENDIX B: CD spectrum

APPENDIX figure 1. The percentage of α-helix during 37°C incubation up to 120 days imitating 1 year storage at room temperature. The decrease of the percentage illustrated the structural deformation during storage.
APPENDIX C: The sensorgrams of liposomes with control antibody

APPENDIX figure 2. Both sensorgrams of plain liposomes and liposomes modified with control antibody (anti-bovine antibody) were found with no change during the injection.
REFERENCES


38. Lofdahl, C.G. and N. Svedmyr, *Formoterol Fumarate, a New Beta-2-Adrenoceptor Agonist - Acute


45. Magder, S., Reactive oxygen species: toxic molecules or spark of life? Critical Care, 2006. 10(1).


52. Thannickal, V.J. and B.L. Fanburg, Activation of an H2O2-generating NADH oxidase in human lung


88. Xie, J., et al., Aminopyridinecarboxamide-based inhaled IKK-2 inhibitors for asthma and COPD:


101. Corvo, M.L., et al., Intravenous administration of superoxide dismutase entrapped in long


178


180. O'Shannessy, D.J., *Determination of kinetic rate and equilibrium binding constants for*


206. Feldman-Billard, S. and E. Heron, *Systemic tolerance of corticosteroid therapy in ophthalmology*


208. !!! INVALID CITATION !!!


231. Kim, H.J., et al., PI3K gamma activation is required for LPS-induced reactive oxygen species...

