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DEVELOPMENT AND EVALUATION OF A SMALL VOLUME LIQUID IMPACTION SURFACE (SVLIS) IN THE NEXT GENERATION IMPACTOR (NGI)

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A THESIS

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ABSTRACT

The growing popularity of inhalation as a route of administration for local action in the lungs as well as systemic effects has necessitated the development of in vitro models for studying the deposition, dissolution and absorption of orally inhaled drug formulations. Models that integrate the study of at least two of these processes would be better able to simulate conditions in vivo. This thesis describes the design and evaluation of a Small Volume Liquid Impaction Surface (SVLIS) in the Next Generation Impactor (NGI), a cascade impactor for evaluation of aerodynamic particle size distribution. This SVLIS modified NGI allowed for incorporation of lung cell cultures or drug dissolution of orally inhaled powders following aerosolization.

A custom SVLIS was designed to accommodate a liquid volume of 5 mL for incorporation in the NGI stages using the gravimetric cup. It allowed the growth of cells in a way as to mimic the thin layer of fluid lining the lung. A549 cells were grown on the SVLIS and saline administration through the NGI did not adversely affect the cells grown on the SVLIS; though airflow alone decreased cell survival.

The SVLIS was incorporated in various stages of the NGI to study the effect of the modification on aerosol deposition patterns of a nebulized model drug. Though significant differences were seen in deposition at some of the stages, the deposition patterns and aerodynamic properties of aerosols obtained in the SVLIS at stage 4 did not differ from those in the unmodified NGI. A model dry powder for oral inhalation was aerosolized through the stage 4 SVLIS modified NGI using a DPI, but the deposition at stage 4 was seen to be different from that in unmodified NGI. A preliminary dissolution study of
powdered theophylline was performed using the SVLIS modified NGI, which showed that
the SVLIS could be potentially used in \textit{in vitro} dissolution models for inhaled drugs.

The results from all the studies showed that the SVLIS has potential applications in
studying inhaled drug absorption and dissolution following deposition in the NGI.
PREFACE

ABSTRACTS:


Dedicated to all my loved ones
ACKNOWLEDGEMENTS

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<tr>
<td>ACI</td>
<td>Andersen Cascade Impactor</td>
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<td>AIC</td>
<td>Air-interfaced culture</td>
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<td>ATI cells</td>
<td>Alveolar type I cells</td>
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<td>DDW</td>
<td>Deionized distilled water</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
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<tr>
<td>DPI</td>
<td>Dry powder inhaler</td>
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<td>ELF</td>
<td>Epithelial lining fluid</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FPF</td>
<td>Fine Particle Fraction</td>
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<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>GSD</td>
<td>Geometric Standard Deviation</td>
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<td>LCC</td>
<td>Liquid-covered culture</td>
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<td>LVLIS</td>
<td>Large volume liquid impaction surface</td>
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<td>MDI</td>
<td>Metered dose inhaler</td>
</tr>
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<td>MMAD</td>
<td>Mean Median Aerodynamic Diameter</td>
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<tr>
<td>MOC</td>
<td>Micro-orifice collector</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>MSLI</td>
<td>Multi Stage Liquid Impinger</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
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<td>NGI</td>
<td>Next Generation Impactor</td>
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<td>PADDOCC</td>
<td>Pharmaceutical Aerosol Deposition Device on Cell Cultures</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic) acid</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SLF</td>
<td>Simulated lung fluid</td>
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<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
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<td>SVLIS</td>
<td>Small volume liquid impaction surface</td>
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<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>VCI</td>
<td>Viable cascade impactor</td>
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CHAPTER 1

Introduction
1. **Introduction**

In the past few decades, there have been many advances in the field of pulmonary drug delivery, both for local action and for systemic effect. The human lungs are a complex organ system in the human body comprising of 300-500 million alveoli and a total surface area approximating 100 m\(^2\) [1]. Thus, the design and testing of formulations for inhalation is challenging because of the various aspects that need to be controlled for delivering drugs to the lung, such as its deposition, dissolution and absorption post inhalation. The anatomical and physiological differences in the different regions of the lung only increase the complexity of the puzzle that is the lung, making inhaled drug formulation development that much more difficult.

![Anatomy of the Respiratory system](attachment:image.png)

**Figure 1:** Anatomy of the Respiratory system [2].
The amount of drug that enters the lung, the location and pattern of drug deposition, residence time of drug at sites of deposition and their absorption into systemic circulation all provide important information to the formulation scientist during development of pulmonary formulations. This information can help determine the extent of pulmonary selectivity of locally acting drugs (e.g. β-agonists) or even used to influence the bioavailability of systemically acting drugs (e.g. proteins and peptides) [3]. The deposition and absorption of aerosolized particles in the lung is affected by anatomical, physiological and pathological factors such as airway geometry [4], lung humidity [5], tidal volumes and flow rates [6], gender [7], inflammation, congestion and constriction of airways and physiological clearance mechanisms [8]. Pulmonary drug deposition is influenced by the kinetics of passive or active absorption into the capillary blood network and non-absorptive clearance mechanisms such as mucociliary clearance, phagocytosis and/or metabolism in the mucus or lung tissue (Figure 2), all of which are lung-region-dependent [9-15].

![Figure 2: Possible routes of elimination of drug from the lungs [15].](image-url)
The development of safe and effective formulations for lung delivery necessitate an understanding of respiratory biopharmaceutics and drug interactions with the lung, apart from the optimization of device and formulation design. Since the lung is more complex than the GIT in terms of lumen access, diverse anatomy, difficulty in drug administration and dosimetry, advances in respiratory biopharmaceutics has been limited [16]. A better ability to assess the mechanisms of deposition and absorption of inhaled drug formulations is essential to advance the understanding of pulmonary biopharmaceutics. Several in vivo, in vitro and ex vivo models have been reported that simulate various anatomical and/or physiological aspects of the lungs in attempts to better understand the effect of the various physiological and pharmacological factors on the absorption patterns and deposition profiles of orally inhaled formulations [1, 12, 17, 18].

Most in vitro models incorporate principles and techniques of cascade impactors to determine aerodynamic size and deposition profiles of orally inhaled formulations [19, 20]. The deposition profiles of drugs in the lungs of live animals can be studied using in vivo imaging techniques such as gamma scintigraphy [21], single photon emission computed tomography (SPECT) [22], positron emission tomography (PET) [23], magnetic resonance imaging (MRI) [24] and fluorescence imaging [25]. However, drawbacks such as higher costs, high radiation doses, safety concerns and requirements of specialized training limit the utilization of these in vivo techniques.

Separate reports have utilized animal models, both small and big animals such as mice, rats, rabbits and dogs, to study the pharmacokinetics and pharmacodynamics of drugs following pulmonary delivery [17, 26]. However, anatomical complexities and inter-species difference between lungs limit some results for human applications [12, 27, 28]. In
contrast, *ex vivo* studies are performed outside the organism in an environment that is artificial yet mimics the biological environment, in order to enable the investigation of isolated physiological processes. Isolated perfused tissue models, like the isolated perfused rat lung model (IPRL) [18], allow the study of drug deposition and absorption in the lung and provide better *in vivo-in vitro* correlation as compared to that obtained from single cell monolayer models [1, 15]. Cell based *in vitro* models have been widely used for studying drug uptake, transport and metabolism by the lungs [29–31], especially since they simulate the microenvironment of lung tissue.

**1.1. Cascade impactors for studying drug deposition in the lung**

Once inhaled, aerosol particles or droplets deposit in different lung regions, as determined by lung morphology, breathing pattern, aerosol morphology and geometry, particle size and density. Aerodynamic particle size is an important factor in determining the regional deposition of aerosolized particles within the respiratory tract, since it can influence the aerosol deposition patterns following inhalation [19, 32–37]. The aerodynamic diameter of a particle is defined as the diameter of a hypothetical spherical particle of unit density (\( \rho = 1.00 \text{ g/cm}^3 \)) that has the same settling velocity as the physical particle [19, 20, 38]. Larger and heavier particles (>5-6 µm) tend to be deposited in the mouth, throat and at points of bifurcation in the early generations of the conducting airways (oropharyngeal deposition). Smaller and lighter particles (~1-5 µm) tend to be deposited in the deeper regions of the lung. The particles smaller than 0.5-1 µm tend to be deposited in the lung through Brownian (random) motion or remain suspended in the airstream and are exhaled. The ideal particle size range to be achieved in order to obtain optimum deposition in the human lung is approximately 2-3 µm [3, 12].
Cascade impactors are the most commonly used instrumentation for the \textit{in vitro} assessment of delivery efficiency both inhalers and nebulizers, since they directly classify orally inhaled particles and droplets according to aerodynamic diameter. Furthermore, impactors measure particle size distribution of the entire drug dose delivered, and not just a random sample of the entire dose. Cascade impactors also allow for the simultaneous quantification of the mass of the active ingredient present in different size ranges in the formulation, independent of any non-drug material present in the sample [20, 39].

Apart from being able to study the deposition pattern of an aerosolized powder, an impactor allows the calculation of the aerodynamic parameters of an aerosol, such as the \textit{Mean Median Aerodynamic Diameter} (MMAD), \textit{Geometric Standard Deviation} (GSD) and the \textit{Fine Particle Fraction} (FPF). They provide an \textit{in vitro} measure of the delivery efficiency of an aerosol. Deposition of aerosol particles on different stages of an impactor can be related, in general terms, to drug deposition in lung regions [40]. These patterns help predict the \textit{in vivo} deposition pattern in a human lung by depositing larger particles in stages corresponding to the upper airway and smaller particles in stages corresponding to the deep lung. Impactors can help predict the deposition pattern of inhaled drug in the respiratory tract; however, they are by no means lung simulators because of geometry at the point of particle impact, collection surface hardness and coating, and operation at constant flow rate in contrast with the variable flow rate in the human lung. It should also be noted that the impactor stages do not directly correlate with any deposition sites in the lung [20].

Cascade impactors operate on the mechanism of inertial impaction, wherein the aerosolized particles are separated on the basis of inertia which is dependent on the particles’ mass and
velocity. Thus, aerodynamic particle size can be determined by keeping the particle velocity constant. Aerosol droplets or particles are drawn through an impactor at a constant air flow rate and separated by according to size on the different stages. The larger and heavier particles with higher inertia settle on the initial stages while the smaller and lighter with lower inertia particles settle on the latter stages [20]. The cut-off diameters for each stage vary depending on the airflow rate through the impactor and are represented as particle sizes with a collection efficiency of 50% [41]. The collection efficiency is determined using the Stoke number which is the ratio of particle stopping distance to the impactor nozzle distance [42]. The particles larger than the cut-off size settle on the collection cup at that stage, while smaller particles are carried to the next stage by the air stream. The various impactors approved by the USP include the Andersen Cascade Impactor (ACI), Multi Stage Liquid Impinger (MSLI) and the Next Generation Impactor (NGI) [20].

1.1.1. Next Generation Impactor (NGI)

![Next Generation Impactor in closed configuration](image)

Figure 3: Next Generation Impactor in closed configuration [43].
The NGI is the impactor that was used in our experiments (Figure 3). It is different from the ACI and MSLI in that it is set up in a horizontal arrangement rather than stacked vertically one above the other. The use of collection cups as impaction surfaces makes for easier handling and makes it easier to design modifications for it [20]. The NGI is found to be operable across a wide range of flow rates and especially preferred for analysis of aerosolized dry powders [19, 39, 44, 45].

Figure 4: NGI in open configuration [43].

The NGI is a planar impactor with seven stages, also referred to as collection cups or impaction surfaces that are removable impaction cups aligned in a horizontal arrangement, with a micro-orifice collector (MOC) at the end (Figure 4). The lower part of the NGI is the base on which is a frame that holds the collection cups and the upper part is the lid that holds in place the seal body containing the eight nozzle pieces (Figure 5) and also contains the inter-stage passageways. The lid attaches to the seal body with the help of two limited torque screws. There are eight nozzle pieces on the seal body, each of which corresponds to each of the seven stages and the MOC. Each of the nozzle pieces are designed to accommodate a specific number and size of holes depending on the stage they correspond
to, except the first stage. There are O-rings surrounding the nozzle pieces which help seal the NGI and prevent any leakage during aerosol testing. The collection cups are tear shaped and the large end of each is located directly below the nozzle corresponding to that stage, and that is where particle impaction occurs. The direction of airflow is from this larger impaction region towards the small end, from where the air is drawn upwards through a cavity in the lid and directed into the next stage [20].

![Figure 5: Seal body of an NGI [43].](image)

The collection cups for stage 1 and the MOC are larger than for the other stages, so as to minimize the impaction of particles near the walls of the cup, which is also known as secondary impaction. The other stages are smaller, since secondary impaction is not a problem at those stages because of the smaller size of the particles that are deposited there. The MOC is mainly responsible for capturing the smaller particles that escape collection on the previous seven stages. An external filter holder is attached downstream to the MOC which contains a glass fiber inserted in it to collect the remaining smaller particles not captured by the MOC. The NGI is closed by the locking the lid with the bottom frame using a past-center cam lock handle mechanism at the front of the NGI.

The induction port is the part of the NGI that is used as the entry point for the aerosolized drug. It is an L-shaped stainless steel tube, one end of which is inserted into the seal body
above stage 1 and the other end is used for attaching the inhalation device like the nebulizer or DPI. It also simulates the human throat in aerosol deposition studies in the NGI. Since its dimensions, specifically the internal diameter govern the amount of drug that enters the impactor, it is designed according to USP specifications. The NGI also comes with an add-on part which is the pre-separator that is mainly used for retention of large particles like those of diluents such as lactose, especially during analysis of dry powder inhalers, without affecting the deposition of the smaller drug particles in subsequent stages. It is attached between the induction port on one end and stage 1 on the other [20, 43].

One of the key parameters in NGI design is the critical impaction distance (Figure 6). It is the distance between the lip of the collection cup and its bottom (the collection surface), that is, the depth of a regular NGI collection cup. The distance ‘b’, which is $14.625 \pm 0.1$ mm for a regular collection cup, needs to be maintained constant in order to achieve consistent deposition profiles of an aerosolized formulation in the NGI.

![Figure 6: Cross-section of an NGI stage. ('b': critical impaction distance)](image)

The logical next step after *in vitro* evaluation of drug deposition using an impactor is usually the testing of the absorption and transport of an inhalable formulation using *in vitro* cell culture models, wherein the cells are treated with the drug formulation in order to study its effects on the cell line. *In vitro* cell culture studies are useful for preliminary toxicity
and absorption testing of drug formulations before progress to ex vivo and in vivo studies [46].

1.2. *In vitro* cell based lung epithelial models

Cell culture techniques have been gaining importance recently as a complement to animal studies for many reasons including their lower cost in contrast with intact animal models, their predictive ability and simulate and their higher and faster throughput. Apart from reduced cost of operation, *in vitro* cell culture techniques ensure reproducibility, robustness, simplicity and better control in performing experiments and in collection of data [1, 12, 16]. Further, humane considerations dictate that the use of animals in research be minimized and replaced with suitable alternatives where possible [47]. However, the major challenge posed to scientists is the development of a universally validated and standardized *in vitro* cell culture model [12].

![Cell culture model](image)

**Figure 7:** *In vitro* cell culture model to uptake, absorption and metabolism across the pulmonary epithelium [1].

The major physical barrier to drug absorption from the lung is the epithelium lining the airway and the alveoli. Attempts to develop cell monocultures that model the cell types found in the majority surface area of both airway (columnar cells) and alveolar epithelium (alveolar type I (ATI) cells) have been underway for the last few decades. Primary cell
cultures of lung epithelium have been developed from species like rats and humans, whereas various immortal human lung epithelial cell lines derived from cancers or transformed from viruses, such as A549, Calu-3, 16HBE14o- and BEAS-2B have been tested for their utility in testing of inhalable drugs [12]. Primary cell cultures of alveolar type II cells freshly isolated from lung tissue, which can differentiate into type I-like cells forming tight junctions that resemble the alveolar epithelium are mainly used to replicate alveolar epithelium since the cell lines available so far are unable to form intercellular tight junctions. However, even though they are similar to the native epithelium, they are far less reproducible, convenient and economical as compared to immortal cell lines, which makes them less suited for absorption studies [15] and the reason why continuous cell lines are far more commonly used for in vitro studies in laboratories.

Continuous cell lines have the benefit of being culture, maintained and propagated using standard media and protocols. These cells can be cryopreserved for experimental flexibility. However, cell models of the lung epithelium often lack features such as intercellular tight junctions, expression of certain receptors and physiological levels of product secretion as seen in healthy in vivo tissues. Thus, the choice of cell line is extremely crucial while designing an in vitro model that mimics the biological barrier [48, 49]. Two continuous cell lines, Calu-3 and 16HBE14o- have been developed as models for the bronchial epithelium, since they are morphologically resemble native bronchial epithelium epithelium and form a physical barrier to drug absorption from the lung [50–52]. Calu-3 cells are derived from the bronchial adenocarcinoma of the airway [53] and 16HBE14o-cells are immortalized and transformed non-carcinoma bronchial epithelial cells obtained from a 1-year old heart transplant patient [54]. Both cell lines have well differentiated
epithelial morphology and tight junctions [1, 16]. A549 is another immortal cell line derived from a human lung adenocarcinoma that is used to replicate the alveolar epithelium since it exhibits characteristics of type II pneumocytes which are present in a small surface area but perform specific functions which makes A549 cells useful in certain studies even though they are unable to form functional tight junctions [55–59]. All three cell lines form confluent lung epithelial cell monolayers in culture, enabling the study of the transepithelial transport kinetics of test molecules, possibly helping predict in vivo absorption of the molecule.

As opposed to primary cell cultures, continuous cells can be cultured onto collagen-coated or untreated permeable filter inserts at lower cell densities. Apart from having been grown in conventional submerged cultures, these cells have been grown under air-interface culture as well, which involves the removal of culture medium from the apical surface on day 2 or 3 of cell growth. The cells are then allowed to reach confluence using media supplied to only the basolateral cell surface, thus allowing aerosol particles to be deposited directly onto semi-dry cell apical surfaces, which is a more realistic replication of aerosol deposition in vivo [60–62].

1.2.1. Aerosolization onto cell cultures

The conventional approach to testing the deposition and absorption of drugs for pulmonary delivery dictates that the deposition profile of the powder/solution be obtained followed by in vitro cell culture studies, wherein the drug solution is directly administered to the cells. However, this approach fails to account for aerodynamic deposition patterns on doses used for cell cultures. This separation of methodologies has contributed to poor correlation between in vitro cell studies and the behavior of some inhaled drug formulations in vivo.
A unification of drug deposition patterns and cell culture methodologies would help create a better model of the human lung in vitro in order to allow scientists to investigate the pharmacokinetics, pharmacodynamics, and toxicokinetics of inhaled drugs. An in vitro model that blends aerosol deposition with its absorption and transport studies would be presumed to be a better predictor of the drug disposition in vivo.

Furthermore, most of the currently used methods for in vitro testing of aerosols involve submersion in physiological buffers/fluids, in order to enable particle separation from the medium, quantification of phenomena like particle dissolution and/or polymer degradation and drug release from polymer particles. However, therapeutic aerosols delivered to the lung impact on the thin layer of fluid lining the lungs. These considerations have necessitated the development of a more physiologically relevant model of the human lungs for aerosol testing [61].

Several reports attempt to unify these approaches for direct aerosolized particle deposition onto cell cultures grown under both submerged and air interface conditions through some form of particle impactor (Table 1). Since deposition in cascade impactor gives a general correlation with drug deposition in the lung regions, specific cell types can be incorporated in specific impactor stages, thereby exposing the cells to particles of in vivo relevance and helping design an in vitro model that closely mimics both drug deposition and transport patterns in the airways.
Table 1: Examples of cell culture studies reported in literature using impactors. (PADDOCC: Pharmaceutical Aerosol Deposition Device on Cell Cultures).

<table>
<thead>
<tr>
<th>Impactor used</th>
<th>Cell line used</th>
<th>Formulation tested</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi stage liquid impinger (MSLI)</td>
<td>Calu-3</td>
<td>Poly(lactic-co-glycolic) acid microparticles</td>
<td>[61]</td>
</tr>
<tr>
<td>Viable cascade impactor (VCI)</td>
<td>Calu-3</td>
<td>Micronized fluorescent marker</td>
<td>[62]</td>
</tr>
<tr>
<td>Multi stage liquid impinger (MSLI)</td>
<td>Calu-3</td>
<td>Salbutamol sulfate and budesonide formulations</td>
<td>[63]</td>
</tr>
<tr>
<td>PADDOCC</td>
<td>Calu-3</td>
<td>Salbutamol sulfate and budesonide formulations</td>
<td>[64]</td>
</tr>
</tbody>
</table>

Fiegel et al. [61] investigated the effect of direct impingement of aerosol particles on Calu-3 cell monolayers grown under conventional liquid-covered culture (LCC) or air-interfaced culture (AIC) conditions. Poly (lactic-co-glycolic) acid (PLGA) microparticles were aerosolized directly onto Calu-3 cells grown on Transwell™ inserts placed under the second stage nozzle of an Astra-type liquid impinger under vacuum at 30 L/min. Studies using sodium fluorescein showed that the permeability of cells grown under LCC conditions was significantly higher after microparticle impingement, in contrast with those under AIC conditions, which remained almost unchanged even after microparticle deposition. This was thought to be a result of a dense mucus coating developed on the cells during the AIC which may have protected the cells during impaction [61].

Cooney et al. [62] also studied the effect of direct aerosol deposition on Calu-3 cells grown under both LCC and AIC conditions, but using a viable cascade impactor to deposit dry powder particles and nebulizer droplets onto cells grown on Transwell™ filter inserts placed on stage 5 of the impactor. They obtained similar results as Fiegel et al. [61] for the
permeability of the fluorescent markers tested, but they were able to show that this *in vitro* model could be applied to nebulizers as well as dry powder inhalers [62].

Bur et al. [63] investigated the transport and absorption of dry powder aerosol formulations of budesonide and salbutamol across Calu-3 cells grown under AIC and LCC conditions by delivering the powder through an MSLI, whereby it was deposited onto cell culture grown inserts placed under the stage 3 nozzle. They demonstrated that dissolution rate of drugs with low solubility governs their rate of absorption in the limited quantity of fluid lining the lung. The study overall showed that the cells grown under AIC conditions are better suited to replicate conditions *in vivo* than those grown under LCC conditions.

Other studies by Hein et al. [64, 65] using devices like the PADDOCC for delivering budesonide and salbutamol dry powder formulations to Calu-3 cells and by Lenz et al. [66] using the ALICE-CLOUD technology to deliver Bortezomib to A549 cells have also helped paved the way for combining aerosol deposition with subsequent absorption studies across cell cultures that model lung epithelial cells.

*In vitro* studies of inhaled drugs would be incomplete without dissolution testing, since the drug has to first dissolve in the fluid lining the lung before it can be absorbed and show its action. *In vitro* dissolution testing can be a reliable and sensitive predictor of *in vivo* bioavailability for some drugs [67]. They are regularly used as part of quality control (QC) studies of commercially manufactured drug formulations for checking batch-to-batch uniformity, stability and detection of manufacturing variations, if any [68].
1.3. *In vitro* dissolution testing of inhaled drugs

The physical state of a drug determines what happens to it immediately after deposition on the lung surface. A free, solubilized drug will quickly diffuse into the epithelial lining fluid (ELF) and become available for absorption, while drug in the form of dry powder particles must dissolve before it can be absorbed and may even be eliminated by clearance mechanisms such as mucociliary or cough clearance before absorption can take place [15]. Dissolution is defined as the process by which a solid substance enters into a solvent to form a solution and is controlled by the affinity between the solid and the solvent. There are official dissolution tests in various pharmacopeias for testing solid and semi-solid dosage forms but not specifically for orally inhaled formulations.

The lung clearance rate of highly hydrophilic compounds is not significantly affected by their dissolution rate, and there are hardly any differences in the pharmacokinetics for different formulation types, unless regional deposition is greatly affected, or if absorption is altered by excipients added to the formulation. The absorption of very poorly soluble compounds which show a fast onset of absorption followed by sustained absorption over time, is assumed to be governed by dissolution rate. The time to achieve peak concentration for micronized lipophilic drugs is believed to be related to their intrinsic solubility [69]. Bur et al. [63] showed through *in vitro* cell culture studies that dissolution of the drug in the fluid layer over the cells was the rate limiting step for the absorption of drug. Assuming the rate of absorption of the drug is very fast, the overall rate of the process is limited by dissolution/drug release.

However, the drug has to dissolve in a very small volume of fluid that is found lining the lung. A total volume of 10-30 mL of liquid is available for dissolution in the human lung.
Its thickness varies from 5-10 µm in the conducting airways and gradually decreases in the distal alveolar region to about 0.01-0.08 µm, though it may be several microns thick in pooled areas [10, 70]. These volumes and thicknesses may be much higher in disease conditions. Thus, it is possible that a drug particle may be completely submerged in the layer of lining fluid in the conducting airway, whereas the particle size maybe much larger than the fluid layer thickness in the alveoli. Thus, the area of solid-liquid interface between the particles and the fluid is proportional to the surface area of the particle in the upper airways but limited by fluid thickness in the alveolar region, leading us to believe that particles deposited in the upper airways might dissolve faster than those in the alveoli. On the other hand, the particles in alveoli may end up dissolving faster due to factors like higher solubility, greater total interfacial surface area and/or faster peripheral absorption [15].

The testing of pulmonary dissolution *in vitro* is a complex task, since each of the parameters affecting dissolution will be different depending of specific lung regions, leading to a variety of different dissolution processes occurring simultaneously, unlike dissolution in the gastrointestinal tract (GIT), which is a continuous process occurring over a series of tanks [71]. Dissolution testing of an inhalable drug would need to take into account the large surface area and small volume of fluid lining the lung surface, which is considered to be mostly stationary. Consequently, knowledge in the area of drug dissolution in the lungs is not as advanced as that of the GIT. As a result, there are currently no *in vitro* dissolution models to help predict *in vivo* release for orally inhaled formulations as opposed to the standard test methods established for oral solid dosage forms [72]. However, several attempts have been made to develop suitable dissolution models [73].
Commercial models like the type 2 dissolution apparatus have been used to study dry powder dissolution by direct placement of the powder in the vessel [74] or into a modified basket to prevent escape directly into the media [75, 76]. Most of the models submerge the powder in large volumes of dissolution medium [67, 68], but models that replicate the air-liquid interface have also been demonstrated [77]. However, not all of these models performed dissolution testing after deposition using a cascade impactor. Samples of correct aerodynamic particle size distribution can be obtained by first deposition of aerosol through a cascade impactor, in order to give a more realistic picture of the size-wise deposition of particles in different lung regions. But the mass of drug per surface area is kept significantly higher, owing to difficulties in quantification otherwise, thereby making the model lose in vivo significance to some extent. In spite of the relative simplicity of in vitro dissolution models compared to the physiologically complex in vivo conditions, a correlation has been obtained in certain cases between in vitro dissolution and in vivo release, especially in case of modified release formulations [15, 78]. Several reports in literature describe attempts made to develop testing methods for aerosol formulations for inhalation after deposition using a cascade impactor [79].

Davies and Feddah [67] developed a model wherein dissolution of three glucocorticoids was studied in a flow-through cell following administration into an ACI, using water, simulated lung fluid (SLF) and modified SLF with L-α-phosphatidylcholine (DPPC) as dissolution media, keeping temperature and flow rate constant. The dose fraction to be used for dissolution was captured using a fiber filter enclosed in a stainless steel holder incorporated in the ACI. This method was able to study dissolution of poorly soluble glucocorticoids in aerosol form. The maintenance of sink conditions and minimization of
sample loading effects due to better dispersion in the fiber filter are some advantages of this model, whereas its drawbacks are the use of non-standard dissolution apparatus and the dependence of dissolution profile on the flow rate [84].

Table 2: Examples of dissolution studies for inhaled drugs in literature [79].

<table>
<thead>
<tr>
<th>Dissolution Apparatus</th>
<th>Aerosol particle collector</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow-through cell apparatus</td>
<td>Fiber filters between membrane filters in stainless steel holders</td>
<td>Linear dependence of dissolution rate with flow rate</td>
<td>[67]</td>
</tr>
<tr>
<td>Franz cell apparatus</td>
<td>0.45 µm microcellulose membrane</td>
<td>Receptor cell connected to 1 L heated vessel</td>
<td>[80]</td>
</tr>
<tr>
<td>Paddle apparatus (USP type 2)</td>
<td>1 µm polycarbonate (PC) membrane</td>
<td>Dissolution rate affected by uniformity of drug loading and particle size</td>
<td>[68]</td>
</tr>
<tr>
<td>Paddle over disk apparatus</td>
<td>NGI dissolution cup</td>
<td>Dissolution rate dependent on drug loading</td>
<td>[81]</td>
</tr>
<tr>
<td>Transwell® system apparatus</td>
<td>0.22 µm polyvinylidene (PVDF) membrane filter</td>
<td>Dissolution rate dependent on filter loading</td>
<td>[82]</td>
</tr>
<tr>
<td>Paddle apparatus (type 2)</td>
<td>Stainless steel filter</td>
<td>Dissolution rate dependent on drug loading/filter orientation</td>
<td>[83]</td>
</tr>
</tbody>
</table>

Arora et al. [82] studied the dissolution of a series of commercial corticosteroid inhaler formulations after their deposition on polyvinylidene fluoride (PVDF) membranes placed at stages 2 and 4 of an ACI. The membrane was then placed in the donor compartment of a Transwell® system with the drug-loaded side facing the receiver compartment and 0.04 mL of medium, either deionized distilled water (DDW) or phosphate buffer saline (PBS), was added to the donor compartment and dissolution profiles obtained at 37°C and 100% humidity. The aim of the study was to study the dissolution of respirable aerosol particles...
in conditions resembling the limited volume of mostly stationary fluid in the lung. One of the advantages of this system is the simulation of \textit{in vivo} conditions due to the diffusion (unstirred) mechanism while some of its drawbacks include that the PVDF membrane is not representative of lung permeability and the extremely small fluid volume leads to lower dissolution and permeation rates at higher drug loading [84].

Son and McConville [81] developed a method wherein they modified different stages of the NGI to include the NGI dissolution cup instead of the regular collection cup. The particles of commercial dry powder inhalers were collected and the insert in the dissolution cup removed, covered by polycarbonate membrane, secured with the membrane holder and dissolution carried out in a conventional dissolution tester using different media like phosphate buffer, PBS and SLF for dissolution. They further carried out optimization of parameters like collection surface, agitation speed and dissolution media using this model. The commercial availability of the NGI dissolution cup, use of standard dissolution apparatus and the ability to analyze particle size fractions on individual stages are some of the advantages of this system, while dependence of dissolution rate on drug loading is a drawback [84].

\textbf{1.4. Hypothesis and Specific Aims}

As previously discussed, an \textit{in vitro} model that integrates aerosol deposition and cell culture testing of drugs for pulmonary delivery would be an improvement in the prediction of \textit{in vivo} drug toxicity and absorption compared to current approaches. Previous studies that [61–63] have demonstrated the feasibility of incorporation of cell cultures in various cascade impactors, have failed to utilize the NGI which has been shown to give more accurate measurements for aerosols over a range of flow rates from 15-100 L/min apart
from being especially preferred for the analysis of dry powder deposition as compared to the ACI or MSLI [19].

Furthermore, there are currently no standardized tests for studying the dissolution of orally inhaled formulations unlike those for oral solid dosage forms, even though dissolution is considered to be an essential quality control tool for evaluation of commercial formulations. Most of the methods that have been developed so far either do not consider that there is a small volume of fluid lining the lung or they use the entire emitted dose for analysis instead of the respirable fraction [67].

The objective of this research project was to design a small volume liquid impaction surface (SVLIS) as a modification to the NGI which would facilitate incorporation of cell cultures in the NGI to enable direct deposition of aerosolized drug on cells. To make sure the modification did not alter the aerodynamic parameters of the drug, aerodynamic parameters aerosolized in the modified needed to be determined and compared with those obtained from an unmodified NGI. Another objective was to develop a dissolution testing method using a model drug to test the extended application of the same modification to better replicate conditions during dissolution in vivo.

The hypothesis of this thesis project was that the incorporation of a SVLIS in the NGI does not affect the aerodynamic deposition profile of aerosolized drugs and can be used for expanded in vitro evaluation of aerosolized drug formulations.

Three specific aims were designed in order to prove this hypothesis. The first aim was to design a SVLIS and incorporate lung cell cultures into the NGI. The second aim involved the evaluation of deposition patterns in the SVLIS in comparison with normal impaction
surfaces. The third aim was to study the dissolution profile of a powdered drug using the SVLIS.
CHAPTER 2

Specific Aim 1: To design a small volume liquid impaction surface (SVLIS) and incorporate lung cell cultures into the NGI using the SVLIS
2. Background

The first step in developing an *in vitro* model was to design a modification to the NGI that would serve as a liquid impaction surface. In order to be useful as a tool for performing *in vitro* cell culture studies and for dissolution testing of orally inhaled drug products, the liquid impaction surface to be designed to replace a regular NGI collection cup needed to fulfill certain requirements. First and foremost, the volume of liquid present should be minimum so as to form a thin liquid layer, because the lung is lined with a thin layer of fluid ranging from a thickness of 0.01-10 µm in different regions [10, 70]. Also, since one of the potential applications of the modification was to be for growing cell cultures, it needed to be designed in a way that visualization of cells under a microscope would be possible.

2.1. Design of the Small Volume Liquid Impaction Surface (SVLIS)

A previously custom designed deep collection cup made of aluminum was previously used in our lab [85] as a modification to be incorporated in stage 4 of the NGI instead of a regular collection cup for incorporation and testing of bacterial cultures. During experiments, this deep cup was filled with 42 mL of liquid in order to maintain the USP specified critical impaction distance ‘b’. The same deep collection cup was initially considered for use in our studies for growing cell culture and dissolution testing. However, it neither fulfilled the requirement of a small volume nor would it be easily possible to grow or visualize the cells in the aluminum cup.

In order to resolve these issues, a new collection surface was developed, an insert which would fit into the commercially available gravimetric cup (MSP Corporation, Shoreview, MN). All the parts of the modification needed to be autoclavable, since cells were to be
grown in them under sterile conditions. The gravimetric cup itself is made out of stainless steel, but the materials chosen for the insert were a plastic called Lexan for the body and glass for its bottom surface, so that it would be transparent and enable visualization under the microscope. The O-rings were made of rubber.

All the materials were tested and were found to show no visible changes after autoclaving them at 121°C for 25 minutes. The dimensions were such as the well would contain 5 mL of liquid when filled to the brim and consequently the critical impaction distance would remain unchanged. Henceforth, this modification will be referred to as the Small Volume Liquid Impaction Surface or the SVLIS and the deep cup modification will be referred to as the Large Volume Liquid Impaction Surface or the LVLIS. The figure 8 shows an unmodified collection cup, the LVLIS and the SVLIS kept side by side and figure 9 shows the schematic cross-sectional representation of all three in order to better understand how the critical impaction distance ‘b’ in the design would be maintained constant even after modification.

The figure 10 shows the detailed schematic design of the SVLIS, consisting of three main parts; the plastic body, the sight glass bottom and the O-rings to hold them together. The metal base attached to the glass bottom with the help of three screws was part of the first prototype, but was later done away with since it was deemed unnecessary for the design. The figure 11 shows the exact dimensions of the SVLIS. All the parts were made to so they could be disassembled and assembled again to form the small volume insert as shown in figure 12.

The insert was designed so as to fit into the gravimetric cup, with the lip of the insert flush with the bottom surface of the gravimetric cup to form an airtight seal. Leak tests were
performed using dye solutions to ensure that there was no leakage when liquid was added after fitting the SVLIS into the gravimetric cup. The figure 13 depicts the assembled configuration of the SVLIS.

![Image of assembled SVLIS configuration](image)

**Figure 8:** Unmodified NGI collection cup (*left*); Large volume liquid impaction surface (LVLIS) (*center*); Small volume liquid impaction surface (SVLIS) (*right*)

![Cross-sectional diagrams](image)

**Figure 9:** Cross-sectional representation of (a) Unmodified NGI collection cup; (b) LVLIS; (c) SVLIS
Figure 10: Design of SVLIS. (a) Cross-sectional view; (b) three-dimensional view

Figure 11: Dimensions of SVLIS (Capacity: 5 mL)
Figure 12: Final design of the SVLIS insert. (a) Top view; (b) Side view

(a)  
(b)

Figure 13: SVLIS insert assembled with the NGI gravimetric cup. (a) Top view; (b) Side view

(a)  
(b)

2.2. Cell growth on SVLIS

The model cell line used for the subsequent studies is the A549 cell line. A549 is a continuous cell line established in 1972 from a human pulmonary adenocarcinoma [57], which expresses the differentiated biochemical properties of type II pneumocytes [55, 56]. Even though type II pneumocytes contribute only to about 10% of the surface area of the deep lung, they are present in a large number and believed to perform distinct functions
like production of surfactant; they are also believed to be progenitor of type II cells and play a role in oxidative metabolism of drugs in the lung [86]. Also, A549 have been shown to be unable form functional tight junctions [58]. As a result, primary cell culture growing in confluent monolayers is most often used to obtain alveolar type I-like epithelial cells. However, A549 cells are accepted as a model for toxicity studies [59, 87–89], since they are fast growing cells that form a confluent monolayer in culture and are easy to handle. Furthermore, A549 cells have been demonstrated to uptake microspheres in the respirable range (~3 µm), making them useful in studying the mechanisms of pulmonary drug delivery of macromolecules and studies have also shown that they are useful in studying clearance of environmental particulates [90, 91]. For the purpose of the following studies, MTT assay was used as a means to determine cell viability after growing cells in the SVLIS and exposing them to airflow and saline in the NGI. The MTT assay is a simple, rapid and sensitive screening procedure usually used for determining drug or excipient toxicity in an in vitro system [92].

2.2.1. Materials

The gravimetric collection cup used in the modification of the NGI (MSP Corporation, Shoreview, MN) was obtained from the MSP Corporation (Shoreview, MN). The glass bottomed insert for the gravimetric cup was obtained custom-made from Benson Machine Works Inc. (Omaha, NE). The A549 cell line obtained from the American Type Culture Collection (ATCC, Rockville, MD) was used for the study. The medium used was Ham’s F-12K medium supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v L-glutamine, 1% v/v non-essential amino acids (NEAA), 1% v/v penicillin-streptomycin solution and 1% v/v sodium pyruvate. Sterile poly-d-lysine for coating the SVLIS was
obtained from EMD Millipore (Billerica, MA). Normal saline obtained from Ricca Chemical Company (Arlington, TX) was sterilized by sterile filtration.

2.2.2. Methods

2.2.2.1. Growth of cells on the SVLIS

The SVLIS was autoclaved to sterilize it and taken inside the cell culture hood for coating its glass bottom. The sterile poly-d-lysine solution was diluted to a final concentration of 100 µg/mL using autoclaved water. 5 mL of this solution was pipetted out on the SVLIS and left overnight in the hood. The SVLIS was placed inside a closed sterile petri dish throughout the course of all experiments to keep it covered in order to maintain its sterility. Just before starting the experiment, the coating solution was removed from the SVLIS and it was washed with the cell culture medium to wash off the excess and ensure uniform coating every time. On day one of the experiment, the cells were passaged and seeded on the SVLIS at the seeding density of 1.068 x 10^6 cells/mL. After plating, the SVLIS was maintained at 37ºC in an incubator at a relative humidity of 85% in an atmosphere of 5% CO₂. The cells were observed under a microscope every day for a week and pictures were taken using a camera attached to the microscope at a magnification of 200x. The medium in the SVLIS was removed and replaced with 5 mL of fresh medium every other day. At the end of every experiment, all the SVLIS components were washed and cleaned thoroughly and soaked in hydrochloric acid before autoclaving to remove any residual coating.

2.2.2.2. Assembly and operation of the NGI

The NGI was assembled as specified in the USP. The induction port was attached to the mouth of the main frame of the NGI. The external filter was fitted into the filter holder
provided to capture droplets that had escaped impaction on the MOC and the entire assembly was connected to a vacuum pump. The airflow inside the NGI was calibrated to 15 L/min using the airflow meter connected to the mouth of the induction port via a custom-made airtight silicone elastomer fitting to maintain the vacuum inside.

2.2.2.3. Incorporation of cells in the NGI

The next step was to study the effect of airflow on the cells when grown on the SVLIS and incorporated in the NGI. For this purpose, the NGI had to be thoroughly cleaned first and sterilized as necessary. Before the start of the experiment, the collection cups, gravimetric cup, the filter assembly and the induction port were sterilized by autoclaving. The airflow meter was surface sterilized using paraformaldehyde by placing it in an airtight chamber overnight. Autoclavable tubing was obtained so that the NGI assembly could be connected to the compressor which is outside the cell culture hood. All the other parts of the NGI, including the base, body and the orifice plate were disinfected using 70% ethanol, before bringing them inside the hood (Figure 14).

Figure 14: NGI cleaned, sanitized and taken inside cell culture hood
On day one of the experiment, the cells were split as usual and plated in six different SVLIS, three as “controls”, again at a seeding density of $1.068 \times 10^6$ cells/mL. Three of these SVLIS were meant to be used as “controls”, meaning that they were not to be exposed to airflow and would be kept in the incubator throughout the course of the study. The three other SVLIS were meant to be used as “samples”, meaning that the cells in them would be exposed to airflow inside the NGI. After 24 hours of plating the cells, that is, on day two of the experiment, the cleaned and sterilized NGI was assembled inside the cell culture hood as instructed in section 3.3.2. The SVLIS containing A549 cells was incorporated in stage 4 of the NGI, since in the past, modifications to the NGI have been primarily carried out on this stage [85]. The NGI was allowed to run at 15 L/min for 10 minutes with the SVLIS containing cells at stage 4. No drug was aerosolized through the NGI and only air was allowed to pass through the system as treatment to the cells. At the end of 10 minutes, the SVLIS containing A549 cells was removed, covered using the petri dish (figure 15) and incubated for 4 hours. After the incubation period, since there was no treatment to be removed, the medium was removed from over the cells and replaced with 5 mL of fresh medium and the SVLIS was incubated for 72 hours. The medium in the SVLIS was removed and replaced with 5 mL of fresh medium every other day.

Figure 15: 'Controls' and 'Samples' plated in the SVLIS placed in petri dishes
After 72 hours of incubation, an MTT assay was performed to determine cell viability. The cells in the SVLIS were treated with 1 mL of MTT solution (5 mg/mL) prepared in Dulbecco’s phosphate buffered saline (DPBS) and incubated for 4 hours. After incubation for 4 hours, the MTT treatment was removed from the cells along with the medium and the cells were then treated with 5 mL a 1:1 solution of 20% w/v sodium dodecyl sulfate (SDS): dimethylformamide (DMF). The SVLIS was then kept inside an incubator-shaker (MaxQ 4450, Thermo Scientific) for an hour at 37ºC. The resultant solution was then pipetted out in triplicates in a 96-well plate and analyzed at 540 nm using the Synergy H1 Hybrid Multi-mode Microplate Reader (Biotek Instruments Inc., Winooski, VT).

After the effect of airflow on cells, the effect of an inert solution, such as normal saline was to be studied on the cells incorporated in the NGI. For this next study, the NGI and its assorted parts were cleaned and sterilized again. This time the Aeroneb Pro nebulizer was also autoclaved. The cells were split and plated in six SVLIS as above, three of which were used as “controls” and no saline was nebulized onto them. The other three were the “samples” and were to be incorporated in the NGI and saline was to be nebulized onto them. The same study as described above was performed by nebulizing normal saline into the NGI containing the cells in the SVLIS at stage 4 for 10 minutes, instead of just passing airflow through it. The subsequent steps performed were also as described above.

Both the experiments were performed in triplicate to enable statistical analysis on the data obtained. Statistical significance was determined using the Student t-test. The cells were observed under the microscope and pictures were taken at 200x throughout the course of the experiments.
2.2.3. Results and Discussion

2.2.3.1. Growth of cells in the SVLIS

The figure 16 shows the growth of the A549 cells in the SVLIS for a period of 6 days, when confluence was reached. From the figure, cells were observed to grow at an expected rate in the SVLIS and it can be inferred that the glass bottom of the SVLIS can be made conducive to the growth of A549 cells.

2.2.3.2. Incorporation of cells in the NGI

After it was established that it was possible to grow cells on the glass bottom of the SVLIS, an attempt was made to incorporate the cells growing on the SVLIS at stage 4 of the NGI. For that purpose, the cells were plated, then placed at stage 4 in the NGI and exposed to airflow for 10 minutes, following which they were allowed to grow. Another experiment
in which saline was aerosolized through the system instead of exposing the cells to airflow only.

Figure 17 shows the cells near confluence as observed under the microscope when exposed to airflow, administered with saline and when untreated (control).

Figure 17: Comparison of cells observed under microscope near confluence; (a) Control; (b) Cells exposed to airflow; (c) Cells treated with saline

The cells appeared to be similar under the microscope in all three cases, that is, there was no apparent change in the physical appearance of the cells. This can be considered to mean that the airflow as well as the saline do not have an obviously adverse effect on how the cells appear.

Figure 18 represents the percent survival of cells in the SVLIS after 72 hours of incubation following exposure to airflow and to saline, as compared to controls that were not exposed to airflow and allowed to grow without disturbance in the incubator. It was observed that the cells that were exposed to airflow showed a significantly (p<0.05) lower percentage survival (70.8%) than the cells used as controls (100%). On the other hand, the cells that were administered saline through the NGI also showed a lower survival rate (87.2%) as compared to control, but the difference was found to be statistically insignificant. There
was also found to be a significant difference between the percent survival of cells exposed to airflow alone and those administered with saline, as can be seen in the graph.

![Cell survival graph](image)

**Figure 18: Cell survival in the SVLIS after 72 hours of treatment**

The results obtained after administration of normal saline as an inert solution to cells incorporated into the NGI with the help of the SVLIS were encouraging, in that the cells showed more or less similar growth to control cells. However, a significant difference was observed between the percentages of cells that survived, when exposed to airflow at the rate of 15 L/min continuously for 10 minutes inside the NGI, as compared to the controls. One reason for this may be that the air flowing through the NGI is causing evaporation of the medium from above the cell layer (studied in detail in section 3.5), thus causing drying out and consequent cell death, since fresh medium is only added to the cells 4 hours after treatment. On the other hand, since nebulization of saline through the system lessens the possibility of evaporation and helps prevent drying out, percentage of surviving cells may be higher than for cells only exposed to airflow.
Thus, the SVLIS may have a potential application especially for \textit{in vitro} testing of nebulized drug formulations. It may also be noted that in a scenario where drug is to be administered using a metered dose inhaler (MDI) or a dry powder inhaler (DPI), the actuation of the inhaler and hence, the time for which the cells would be exposed to high airflow rates, would be a few seconds at most. That might minimize cell death due to evaporation of medium. However, more studies need to be performed using MDIs and DPIs at the pertinent airflow rates in order to obtain conclusive evidence regarding the same. Those studies are not in the scope of this thesis and will not be discussed in further detail. Meanwhile, it can be inferred from the available evidence that using the SVLIS to incorporate cell cultures in the NGI is a promising new method for \textit{in vitro} testing of orally inhaled formulations.

\textbf{2.2.4. Summary of preliminary studies}

\textbf{2.2.4.1. Cell growth in LVLIS}

The modification to the NGI previously used in our lab was the aluminum deep collection cup or the large volume liquid impaction surface (LVLIS) to replace the regular collection cups. The LVLIS was initially considered for use as a tool for \textit{in vitro} testing of inhalable formulations by using it to incorporate cell cultures in the NGI. Consequently, an attempt was made to study the effect of exposing A549 cells to this LVLIS before eventually developing the SVLIS.

\textbf{2.2.4.2. Materials}

Deep collection cups machined from block aluminum were obtained custom made from Benson Machine Works Inc. (Omaha, NE). Sterile petri dishes were obtained from Fisher
Scientific (Pittsburgh, PA). The medium was prepared and sterilized as described in section 2.2.2.1.

2.2.4.3. Methods

Two of the deep collection cups (LVLIS) were sterilized by autoclaving before starting the experiment. The cells were passaged and 42 mL of the resulting cell suspension was used as sample for exposure of the cells to the aluminum plate and the petri dish. 42 mL each of the cell suspension was poured into the LVLIS and the petri dish, which was used as control. The suspension was poured into both the LVLIS and the petri dish, allowed to sit for 15 minutes before plating them in a six well plate in triplicate as well and both sets of cells (control and sample) were incubated. At the end of 96 hours, an MTT assay was performed as described in section 2.2.2.1 to determine cell viability.

2.2.4.4. Results and Discussion

Figure 19 represents the growth of cells (percent survival) following incubation for 96 hours after exposing the cells to the aluminum LVLIS for 15 minutes, as compared with cells exposed to regular sterile petri dishes for the same amount of time as control. The cells that were exposed to the aluminum SVLIS for 15 minutes before incubation for 96 hours (4 days) showed a survival rate only slightly less (93.6%) than the controls exposed only to a plastic petri dish (100%), but the difference between the two was not significant (p>0.05).
Even though the results obtained from the above study were encouraging, there were certain drawbacks associated with using the LVLIS to modify the NGI for use in \textit{in vitro} cell culture studies. In order to function at the correct critical impaction distance \textquote{b}, the LVLIS needs a large quantity of liquid (42 mL) to be added to it. This would not simulate the conditions in a healthy human lung since a small volume of fluid is present lining the lungs and the thickness of fluid lining in most areas is not more than 10 µm and in some areas, it may be as low as 0.08 µm [10, 70]. Secondly, the metallic nature of the LVLIS made it difficult to actually grow the cells on its bottom surface and even more difficult to visualize them under a microscope due to the opacity of its bottom surface. That was the rationale behind only exposing the cells to the LVLIS and then plating them in a plastic six-well plate, rather than attempting to grow the cells on the metal directly. Thus, in order to overcome these drawbacks, the SVLIS was designed to have a glass bottom to enable cell visualization and to contain a smaller volume of liquid (5 mL).
CHAPTER 3

Specific Aim II: To evaluate the deposition patterns in a SVLIS compared to normal impaction surfaces
3. Background

For the purpose of this thesis project, the NGI was modified to serve as an enhanced in vitro testing model. It was critical to ensure that any modification introduced into the NGI would not affect the deposition and aerodynamic parameters of the formulation aerosolized through the NGI. It was also necessary to compare these properties in the modification with those of drug aerosolized through unmodified NGI. This section describes the evaluation of aerosol deposition patterns and aerodynamic properties the SVLIS modified NGI.

3.1. Selection of model drug

![Figure 20: Structure of theophylline [93]](image)

The primary purpose of this aim was to study and compare the aerodynamic parameters and deposition patterns of an aerosol nebulized through an unmodified NGI with those from a SVLIS modified NGI. Theophylline was chosen as a model drug for use in nebulized solutions. Theophylline, chemically known as 1, 3-dimethylxanthine, is a white, odorless, crystalline, bitter tasting powder with a molecular weight of 180.164 Da (Figure 20). Theophylline is a bronchodilator used to relieve airflow obstruction in acute asthma patients and to reduce the severity of symptoms in patients suffering from chronic asthma. It is soluble in water up to a concentration of 8.3 g/L at 20°C and has a distinctive ultraviolet
(UV) absorbance with maximum absorbance ($\lambda_{\text{max}}$) at a wavelength of 273 nm. All these factors made theophylline a sound choice for the model drug.

### 3.2. Method for detection of theophylline

#### 3.2.1. Materials

Anhydrous theophylline was obtained from the City Chemical Corporation (New York, NY). A PURELAB Ultra water purification system (ELGA LabWater, Woodridge, IL) was used to obtain the deionized water. A Synergy H1 Hybrid Multi-mode Microplate Reader (Biotek Instruments Inc., Winooski, VT) was used for the detection of theophylline and data analysis was performed using the Gen5 Data Analysis Software (Biotek Instruments Inc., Winooski, VT). 96-well plates obtained from Corning Inc. (Corning, NY) were used for detection of theophylline in the microplate reader.

#### 3.2.2. Methods

A theophylline stock solution was prepared by dissolving 25 mg of theophylline in 50 mL tap water, to produce a solution of concentration 500 µg/mL. Dilutions were made from the stock using deionized water in a ratio of 1:3 for preparations of a standard curve of theophylline. The Gen5 Data Analysis software was used to create a protocol for setting the study parameters. The detection method was set so as to measure the absorbance of the sample solutions at a wavelength of 273 nm ($\lambda_{\text{max}}$ of theophylline). The blanks, standards and samples were assigned appropriate names in the “plate layout” section. The “Transformation” section was set to subtract each well from the blank well to negate effects from any contaminants.
200 µL of each of the dilutions and the blank (deionized water) was pipetted into the 96-well plates in triplicate. The absorbance of the solutions were obtained by reading the plate in the plate reader at 273 nm using the aforementioned protocol. A standard curve was obtained by plotting the curve of absorbance against the known concentrations of each dilution. The concentrations of unknown solutions were determined using their absorbance by extrapolation on this curve.

3.2.3. Results and Discussion

A linear standard curve was obtained by plotting a graph of absorbance vs concentration. The plate reader was found to be able to detect concentrations of theophylline between 55.556 µg/mL and 0.008 µg/mL (Figure 21). The curve was plotted and the equation found using the Microsoft Excel software. A correlation coefficient ($R^2$) value of 1 was obtained indicating a goodness of fit of the line. The slope and intercept of the line was calculated and then the values used to calculate the concentration of unknown solutions using equation 1 as under:

\[
\text{Concentration of Unknown solution} = \frac{(\text{Absorbance of Unknown solution} - \text{Intercept})}{\text{Slope}} \quad (1)
\]

These results indicated that it is possible to detect and quantify theophylline using its UV absorbance in a plate reader. The method was also found to be reproducible, ensuring consistency in results obtained in future experiments.
### 3.3. Validation of Unmodified NGI

#### 3.3.1. Materials

Anhydrous theophylline was obtained from the City Chemical Corporation (New York, NY). The PURELAB Ultra water purification system (ELGA LabWater, Woodridge, IL) was used to obtain the deionized water. The theophylline solution was aerosolized into the Next Generation Impactor (MSP Corporation, Shoreview, MN) using an Aeroneb Pro nebulizer (Aerogen Ltd., Galway, Ireland). The airflow rate in the NGI was calibrated using the TSI 4000 series airflow meter (TSI, MN, USA). A glass fiber filter (MSP Corporation, Shoreview, MN) was attached to the NGI to entrap any droplets that escaped impaction on the last collection cup (MOC). A Synergy H1 Hybrid Multi-mode Microplate Reader (Biotek Instruments Inc., Winooski, VT) was used for the detection of theophylline and data analysis was performed using the Gen5 Data Analysis Software (Biotek Instruments Inc., Winooski, VT). 96-well plates obtained from Corning Inc. (Corning, NY) were used to hold the samples for detection.
3.3.2. Methods

3.3.2.1. Preparation of theophylline solutions

5 mL of the stock solution prepared as described previously in section 3.2.2 (500 µg/mL), was used as the sample solution for nebulization into the NGI. Tap water was used since the traces of ions present in the tap water are essential for nebulizing the drug.

3.3.2.2. Assembly of the NGI

The NGI was assembled and operated as specified in section 2.2.2.2. The airflow inside the NGI was calibrated to 15 L/min using the airflow meter connected to the mouth of the induction port via a custom-made airtight silicone elastomer fitting to maintain the vacuum inside.

3.3.2.3. Nebulization of theophylline solution

The Aeroneb Pro nebulizer was set up so as to enable entry of the aerosol into the NGI through the induction port due to the airflow movement. 5 mL of the theophylline stock solution (corresponding to 2.5 mg of theophylline) was pipetted out into the head of the nebulizer. The control unit and the vacuum pump was switched on and the theophylline solution was allowed to nebulize through the NGI till dryness for a period of 10 minutes.

3.3.2.4. Recovery and Analysis of nebulized theophylline

The theophylline solution and droplets deposited on each component of the NGI (head, neck of nebulizer, induction port, stages 1-7, orifice plates and filter) were collected following aerosolization. The collection was carried out in separate volumetric flasks assigned to each component by washing each of the components at least thrice with
deionized water and then the volume in each flask was made up to 100 mL using deionized water. The sample recovered from the NGI filter was filtered using a 0.2 µm filter, so as to clear the solution of any glass fibers. Each nebulization was performed in triplicate.

Following the procedure described in section 3.2.2., each of the collected samples was pipetted out in triplicate in a 96-well plate and read using the Synergy H1 Hybrid Multimode Microplate Reader and subsequently analyzed using the Gen5 Data Analysis Software. A standard curve was also run along with the sample as described in section 3.2.2 to enable determination of the unknown concentrations. The amount of drug deposited in the induction port and the NGI is defined as the Emitted Dose (ED). The amount of drug deposited in the NGI (% recovery) was calculated using equation 2:

\[
\text{Percent Dose recovered} = \frac{(\text{Amount of drug recovered from the nebulizer and NGI})}{(\text{Amount of drug aerosolized})} \times 100 \quad (2)
\]

The aerodynamic parameters, namely the Fine Particle Fraction (% FPF), the Mass Median Aerodynamic Diameter (MMAD) and the Geometric Standard Deviation (GSD) were then calculated according to guidance in the USP. The % FPF (≤ 5µm) represents that portion of the particles deposited in the NGI having a diameter less than 5 µm. The MMAD is the diameter above and below which 50% of the particles exist [1, 94]. The GSD characterizes the variability of particle size distribution. The aerodynamic particle size distribution of the powder can be characterized by the MMAD and GSD and they were calculated using the equation of the % cumulative recovery against the aerodynamic diameter as described in the USP [43]. The above study was performed in triplicates each time and compared with a similar study performed for the modified NGI.
3.3.3. Results and Discussion:

Figure 22: Deposition pattern of nebulized theophylline in unmodified NGI

The MMAD of the theophylline solution was found to be 3.54 ± 0.07 µm, its GSD was found to be 3.17 ± 0.27 µm and the FPF was found to be 65 ± 1.1%. This FPF value reflects a large cumulative deposited dose based on the deposition pattern of theophylline in the unmodified NGI (Figure 22). These values are representative of the readings that were expected to be obtained from all future studies.

The MMAD value of 3.54 µm lies between the desirable size range of 1 to 5 µm [1, 95, 96], which is sufficient for deposition of the drug in the lower bronchioles and alveoli. The MMAD value of 3.54 µm lies between the desirable size range of 1 to 5 µm. The GSD obtained should be smaller than 1.3 and ideally closer to 1.2[44]. The GSD value of 3.17 µm was slightly on the higher side, indicating the polydisperse nature of the aerosolized particles. Usually, a narrow particle size distribution is known to be produced due to aerosolization by vibrating mesh nebulizers. However, the formulation was not optimized to yield uniform particle sizes prior to use which might be the major contributing
factor to a higher GSD. Besides, nebulizer performance and efficiency might play a role. The aerodynamic diameter of approximately 65% of the nebulized theophylline recovered from the NGI was found to be below 5 µm.

The MMAD obtained was found to be desirable, whereas the GSD was slightly higher, indicating polydispersity of the particles. The aerodynamic deposition pattern and the aerodynamic parameters obtained in this study were meant to serve as the baseline values for comparison against values obtained from all future studies. However, studies performed previously in the same lab [85] suggested that results obtained from different studies performed varied to a certain extent, probably due to changes in nebulizer performance over the period of time that passed between each individual study. Keeping that in mind, a validation study was performed on the unmodified NGI on the same day each time a similar study was performed on the modified NGI, so as to nullify any variations due to these changes in nebulizer performance. This procedure was followed for all the repetitions of the studies (n=3).

3.4. Validation of the modified NGI

The SVLIS modified NGI was developed with the intent to provide a liquid collection surface for aerosol particles/droplets that could be a close approximation of the thin liquid layer present lining the lung. Also, this liquid layer was meant to be utilized for growing cell cultures to mimic the physiological environment of the lung to some extent (section 2). Stage 4 was initially chosen as the prototype stage for studying the effect of airflow and saline administered through the NGI on A549 cells. However, the subsequent studies attempt to determine the applicability of this modification to other NGI stages as well, namely stage 4, 5, 6 and 7, since the cut-off diameters at these stages lie between the
respirable range of 1-5 µm, by comparing the deposition profiles and aerodynamic parameters obtained by modifying those stages with those of unmodified NGI.

3.4.1. Materials

The gravimetric collection cup used in the modification of the NGI (MSP Corporation, Shoreview, MN) was obtained from the MSP Corporation (Shoreview, MN). The glass bottomed insert for the gravimetric cup was custom designed and fabricated (Benson Machine Works Inc., Omaha, NE). The other materials used, including the drug (theophylline) were as described in section 2.3.1.

3.4.2. Methods

A theophylline stock solution was prepared as described in section 3.2.2. The glass bottomed insert was fitted into the gravimetric cup to make the SVLIS. 5 mL of deionized water was added to the SVLIS so that it was filled to the brim to keep the critical impaction distance (distance between the surface of water and the lip of the gravimetric cup) unchanged. The assembled SVLIS was then incorporated in stage 4 of the NGI to replace the regular collection cup at that stage. The NGI was assembled and the theophylline solution was nebulized through it as described in section 2.3.2. Drug was recovered and analyzed as previously described. The same experiment was repeated with the unmodified NGI on the same day as the modified.

Further, the same study was performed by subsequent incorporation of the SVLIS in stage 5, 6 and 7 of the NGI in turn, in exactly the same manner as described above. All the studies were performed in triplicate to allow for statistical analysis and comparison of drug deposition and aerodynamic parameters.
The percentage of drug recovered and the aerodynamic parameters (MMAD, GSD and FPF) were calculated as described in section 3.3.2 and compared with those obtained from the unmodified NGI. The deposition pattern was also determined and compared with that obtained from the NGI. The percent of theophylline deposited at each stage gave an idea of the in vitro deposition efficiency. Statistical significance (p<0.05) was calculated by performing student t-tests on the data to compare the two data sets (unmodified and modified).

3.4.3. Results

3.4.3.1. Incorporation of SVLIS at Stage 4

![Figure 23: Stage-wise deposition for unmodified NGI and NGI with SVLIS at stage 4 (n=3). Blue columns (left): Deposition in unmodified NGI; Red columns (right): Deposition in modified stage 4](image)

Following nebulization, theophylline deposition patterns were evaluated in both the unmodified NGI and modified NGI with SVLIS at stage 4 (Figure 23). Stage 4 is highlighted in the figure to indicate modification. No significant difference was found
between the deposition in unmodified and modified NGI at any of the stages after performing statistical tests (Student t-test). The aerodynamic parameters for theophylline obtained after nebulization through the unmodified and modified NGI were as under (Table 3). The aerodynamic parameters for theophylline (MMAD, GSD and FPF) obtained from nebulization into the modified NGI were also statistically equivalent to those obtained from unmodified NGI.

Table 3: Aerodynamic parameters for unmodified NGI and NGI with SVLIS at stage 4 (n=3)

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI (n=3)</th>
<th>SVLIS at Stage 4 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>3.54 ± 0.60</td>
<td>3.59 ± 0.20</td>
</tr>
<tr>
<td>GSD</td>
<td>3.17 ± 0.27</td>
<td>3.25 ± 0.43</td>
</tr>
<tr>
<td>FPF≤5µm (%)</td>
<td>65.00 ± 1.10</td>
<td>62.70 ± 5.20</td>
</tr>
</tbody>
</table>

3.4.3.2. Incorporation of SVLIS at Stage 5

Figure 24: Stage-wise deposition for unmodified NGI and NGI with SVLIS at stage 5 (n=3). Blue columns (left): Deposition in unmodified NGI; Green columns (right): Deposition in modified stage 5. * = significant difference
The next modification was the incorporation of the SVLIS at stage 5 in order to provide a proof of concept of the utility of the modification in other stages of the NGI where the aerodynamic cutoff is below 5 µm at the airflow rate of 15 L/min. The above graph (figure 24) compares the percentage of deposited dose at each stage of the modified NGI containing the SVLIS at stage 5 with that obtained from unmodified NGI. Stage 5 is highlighted to indicate modification. A statistically significant difference was found between the deposition in the unmodified and modified NGI at stages 5 and 7. The table 4 below shows the aerodynamic parameters obtained by nebulizing theophylline through unmodified NGI and modified NGI with SVLIS incorporated at stage 5. Even though the deposition pattern showed a difference at two of the stages, there was no significant difference between the aerodynamic parameters obtained from the unmodified and modified NGI.

Table 4: Aerodynamic parameters for unmodified NGI and NGI with SVLIS at stage 5 (n=3).

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI (n=3)</th>
<th>SVLIS at Stage 5 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>3.85 ± 0.02</td>
<td>3.90 ± 0.04</td>
</tr>
<tr>
<td>GSD</td>
<td>2.60 ± 0.11</td>
<td>2.64 ± 0.12</td>
</tr>
<tr>
<td>FPF_{≤5 µm} (%)</td>
<td>62.90 ± 0.70</td>
<td>61.30 ± 1.30</td>
</tr>
</tbody>
</table>
3.4.3.3. Incorporation of SVLIS in Stage 6

![Graph showing stage-wise deposition for unmodified NGI and NGI with SVLIS at stage 6 (n=3). Blue columns (left): Deposition in unmodified NGI; Pink columns (right): Deposition in modified stage 6. * = significant difference.]

The next modification involved the incorporation of SVLIS at stage 6. The above graph (figure 25) is a comparison of the deposited doses of theophylline at each stage obtained from the unmodified and modified NGI containing SVLIS at stage 6, which is highlighted in the figure. A statistically significant difference was found between the deposited doses in the unmodified and modified NGI at stages 6 and 7. The table 5 below shows the aerodynamic parameters obtained after nebulization of theophylline into the unmodified and modified NGI with SVLIS at stage 6. Again, it was found that there was no significant difference in the aerodynamic parameters obtained from the modified NGI as compared to the unmodified NGI.
Table 5: Aerodynamic parameters for unmodified NGI and NGI with SVLIS at stage 6 (n=3).

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI (n=3)</th>
<th>SVLIS at Stage 6 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>3.47 ± 0.39</td>
<td>3.47 ± 0.38</td>
</tr>
<tr>
<td>GSD</td>
<td>2.44 ± 0.25</td>
<td>2.71 ± 0.09</td>
</tr>
<tr>
<td>FPF≤5 µm (%)</td>
<td>64.00 ± 0.30</td>
<td>64.90 ± 1.90</td>
</tr>
</tbody>
</table>

3.4.3.4. Incorporation of SVLIS at Stage 7

Figure 26: Stage-wise deposition for unmodified NGI and NGI with SVLIS at stage 7 (n=3). Blue columns (left): Deposition in unmodified NGI; Orange columns (right): Deposition in modified stage 7. *=significant difference

In this last modification, the SVLIS was incorporated at stage 7. The above graph (figure 26) compares the deposition obtained at each stage of the unmodified and modified NGI containing SVLIS at stage 7, after nebulizing theophylline through it. Stage 7 is highlighted to indicate modification. In this case, deposition at three stages (1, 2 and 7) differed significantly in the modified NGI as compared to the unmodified NGI. The aerodynamic parameters obtained by nebulizing theophylline into the unmodified and modified NGI
with SVLIS at stage 7 were as under (table 6). For the first time, a significant difference was obtained in the MMAD of the modified NGI from that of the unmodified NGI, though the other two parameters (GSD and FPF) remained statistically equivalent.

Table 6: Aerodynamic parameters for unmodified NGI and NGI with SVLIS at stage 7 (n=3); * = significant difference.

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI (n=3)</th>
<th>SVLIS at Stage 7 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>3.85 ± 0.02</td>
<td>4.09 ± 0.04*</td>
</tr>
<tr>
<td>GSD</td>
<td>2.60 ± 0.11</td>
<td>2.47 ± 0.08</td>
</tr>
<tr>
<td>FPF≤55 µm (% )</td>
<td>62.90 ± 0.70</td>
<td>60.80 ± 1.80</td>
</tr>
</tbody>
</table>

3.4.4. Discussion

The results showed that the incorporation of a liquid impaction surface at stage 4 using a small volume insert did not affect the deposition pattern or the aerodynamic parameters of the drug. One of the advantages of this modification was that it minimized dead spaces in the collection cup and since all it involved was adding water to the SVLIS, it was convenient to achieve. Further, the solubility of theophylline in water made the process of its collection and quantitation from the stage simpler. The possibility of variations in nebulizer performance was eliminated by performing the study on the unmodified NGI on the same day as that on the modified NGI.

In the above experiments, except when the SVLIS was incorporated in stage 4, a significant difference was observed in the deposited dose at two or more of the stages of the modified NGI as compared to the unmodified NGI. It was also observed that the modified stage (for instance stage 5 when SVLIS was incorporated in it) was one of the stages showing the difference. In each case, the deposition in the modified stage was found to be less than that
same stage in the unmodified NGI. Also, deposition at stage 7 differed significantly each time.

Some of the factors that might be affecting the drug deposition in the NGI stages are particle size, particle density, airflow inside the NGI, re-entrainment of particles and the critical impaction distance. In our experiments, the theophylline used was the same throughout and solutions were prepared from it following the exact same procedure every time. Also, the studies on unmodified and modified NGI were performed on the same day. Thus, the possibility of changing particle size being the reason for varied results in aerosol deposition in the unmodified and modified NGI is unlikely. The same can be said for particle density, since the theophylline used was the same and was stored in the manner throughout. Further, all the experiments were performed under constant laboratory conditions, without substantial changes in temperature, atmospheric pressure or relative humidity, minimizing the possibility of change in powder density through aggregation.

The airflow rate inside the NGI is calibrated to remain constant at 15 L/min throughout the experiment. However, in case of the modified NGI, the air encounters liquid (water, in this case) at the stage where SVLIS is incorporated, while traversing through the NGI. It was not possible account for exact variations in localized airflow patterns that might be affecting the air movement downstream from the modified stage. One approach to minimize this problem might be to use a gel or higher viscosity fluid instead of water as the collection surface in future studies.

Re-entrainment can be explained as the bouncing of the particles off the surface of the collection cups on impact and their subsequent settling on the orifice plate above or moving on to the latter stages. The collection cups were not coated with Sigmacote (silicone in
heptane) to prevent this re-entrainment as suggested in the USP, since erratic results were obtained in the preliminary studies performed after coating. Thus re-entrainment is not likely one of the reasons for discrepancies observed in the deposition; however, the chances of re-entrainment are minimum at the modified stage, since the particles impact on the liquid surface of the SVLIS. There is a slight possibility that re-entrainment may be occurring from the stages before the modified stage, though a difference in deposition at the initial stages (stage 1 and 2) was only found when stage 7 contained the SVLIS.

The deposition will most certainly be affected if the critical impaction distance (distance b) changes to a great extent over the span of nebulization (10 minutes). There is a high possibility of this variation in this case, since the impaction surface is a liquid (water) and losses due to evaporation might result in a considerable loss in height of the liquid, thus affecting distance b. If losses due to evaporation are sufficiently high, the height of the liquid in the SVLIS will reduce, causing the distance b to increase. This would mean the particles will have a longer distance to travel for impaction, causing smaller and lighter particles to not impact on that stage and instead move with the airstream towards the successive stages. This would explain why the deposition at the modified stage is affected and is significantly lower than at the same stage in an unmodified NGI. It would also explain the higher deposition at some of the stages subsequent to the one containing the SVLIS. However, this theory only holds if a higher deposition is obtained in stages after the modification as compared to the same stages in unmodified NGI.

In order to confirm whether there is a significant change in the height of liquid, and in turn, the critical impaction distance during aerosolization, a study was performed to determine
the losses due to evaporation at different airflow rates and using two different liquids in the SVLIS (deionized water and cell culture medium).

3.5. Validation of modified NGI using Dry Powder Inhaler (DPI)

The results obtained from the study described in section 3.4.3 directly leads to testing the application of the SVLIS in the NGI for dry powder formulations. Besides, one of the potential applications of the SVLIS is proposed to be in dissolution testing of orally inhaled dry powders, which necessitated that the deposition of a DPI in the modified NGI be compared to its deposition in an unmodified NGI. Since encouraging results were obtained when SVLIS was incorporated at stage 4 of the NGI, stage 4 was chosen for this study as of this point.

3.5.1. Materials

Anhydrous lactose was obtained from Sigma-Aldrich Co. (St. Louis, MO). The Sylgard ® 184 silicone elastomer kit (Dow Corning, MI, USA) was used to make a custom mouthpiece adapter for maintaining an airtight connection between the DPI and induction port. The empty hard gelatin capsules were obtained from Gallipot Inc. (St. Paul, MN). A DPI used for the study was the Foradil Aerolizer (Merck and Co., NJ). A Planetary Ball Mill PM 100 (Retsch Inc., Newtown, PA) was used for dry powder homogenization. The gravimetric collection cup used in the modification of the NGI (MSP Corporation, Shoreview, MN) was obtained from the MSP Corporation (Shoreview, MN). The glass bottomed insert for the gravimetric cup was obtained custom-made from Benson Machine Works Inc. (Omaha, NE). The other materials used, including the drug (theophylline) were as described in section 2.3.1.
3.5.2. Methods

250.3 mg of theophylline along with 4.7557 g of lactose were homogenized using the ball mill (three cycles of 30 minutes each at 250 rpm). 100 mg of this uniformly mixed powder was filled in each of the hard gelatin capsules so that each capsule contained 5 mg of theophylline. The exact weights may vary between capsules. The NGI was assembled as described in section 2.3.2 and airflow was calibrated to 60 L/min. A custom mouthpiece adapter was made using the Sylgard ® 184 silicone elastomer kit in order to maintain an airtight connection between the DPI and the induction port. The mouthpiece end of the DPI, when inserted into the adapter, must align with the horizontal axis of the induction port in the same orientation as intended for use and the front face of the mouthpiece should be flush with the face of the induction port, forming an airtight seal [43]. The capsule was inserted inside the DPI, punctured and the DPI was actuated for 30 seconds under vacuum. Drug was recovered and analyzed as previously described. The data obtained was used for calculation of deposition pattern as well as the aerodynamic parameters (MMAD, GSD and FPF). The study was repeated using the modified NGI containing SVLIS at stage 4 instead of a regular collection cup for comparison. As mentioned in section 3.4.2, the experiment with the unmodified NGI was performed on the same day as the modified. The above study was performed in triplicate to allow for statistical analysis and comparison of drug deposition and aerodynamic parameters.
### 3.5.3. Results and Discussion

Figure 27: Stage-wise deposition for unmodified NGI and NGI with SVLIS at stage 4 for DPI (n=3). Blue columns (left): Deposition in unmodified NGI; Purple columns (right): Deposition in modified stage 7. *= significant difference

The above graph (figure 27) gives a comparison of the deposition of theophylline obtained from each stage of the unmodified NGI with that of the modified NGI containing SVLIS at stage 4, when powdered drug was aerosolized through the NGI using a DPI. Stage 4 is highlighted to indicate modification. A significant difference was observed between the deposited dose in the unmodified and modified NGI at stage 4 only. The aerodynamic parameters obtained after the aerosolization of DPI containing theophylline into the NGI are represented in the table 7 below. No significant difference was observed in the aerodynamic parameters obtained from the modified NGI as compared to the unmodified NGI.
Table 7: Aerodynamic parameters for unmodified NGI and NGI with SVLIS at stage 4 for DPI (n=3).

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI (n=3)</th>
<th>SVLIS at Stage 7 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>5.85 ± 0.22</td>
<td>5.66 ± 0.09</td>
</tr>
<tr>
<td>GSD</td>
<td>1.46 ± 0.09</td>
<td>1.55 ± 0.05</td>
</tr>
<tr>
<td>FPF≤5 µm (%)</td>
<td>8.76 ± 2.00</td>
<td>10.74 ± 1.22</td>
</tr>
</tbody>
</table>

As stated previously, the collection cups were not coated with silicone before performing any of the studies. That might have led to re-entrainment of the powder or ‘particle bounce effects’, which may be responsible for the difference in deposition observed between stage 4 of the modified and unmodified NGI. That might explain the fact that a slightly lower deposition was obtained in the initial stages of the modified NGI, while it was higher at stage 4, which was the modified stage containing the SVLIS. At this stage, the particle impaction is on water instead of a solid surface, which is why re-entrainment might not have occurred at stage 4 itself. The USP and European Pharmacopeia both recommend coating of the collection cup surfaces with silicone oil to reduce particle bounce and inter-stage loss, particularly for testing of DPI formulations [97]. Thus, an approach that might help overcome this problem may be to coat the collection surfaces with the Sigmacote and repeat the study.

3.6. Evaluation of effect of airflow on liquid height in the SVLIS

Evaporation losses from the surface may result in a change in weight as well as height of a liquid. There is a possibility that the height of liquid in the SVLIS may decrease during nebulization due to losses from evaporation of liquid under the effect of airflow, consequently affecting the critical impaction distance. This necessitated the evaluation of
the effect of airflow on the liquid in the SVLIS and its effect on the drug deposition pattern in the NGI.

3.6.1. Materials

The materials used were as described in section 2.3.1. Ham’s F-12K medium was prepared as instructed in section 2.2.1.

3.6.2. Methods

For this study, two of the SVLIS were filled with 5 mL of deionized water. One SVLIS was kept on the countertop to serve as ‘control’ and the other was incorporated in different stages of the NGI in turn as the ‘sample’. The NGI was assembled and operated as described in section 2.3.2, only for these experiments no drug was aerosolized through the NGI and air alone was allowed to circulate through the system. The SVLIS were weighed at the beginning of the experiment (Time 0) and after specific time intervals of 1, 2, 3, 4, 5, 10 and 15 minutes. Since the loss in weight of the water is proportional the loss in height, height loss after each time interval was accordingly calculated using the following equation 3.

\[
\text{Percent loss in height} = \frac{\text{Loss in liquid height in mm}}{\text{Total height of regular collection cup}} \times 100 \quad \text{(3)}
\]

The percent loss values were calculated as a percentage of the total height of a regular NGI collection cup since that corresponds to the critical impaction distance ‘b’. The same study was performed at two other airflow rates, 30 L/min and 60 L/min, with incorporation of the SVLIS at different stages in each case. The stages at which the SVLIS would be incorporated were chosen based on the cut-off diameters for the stages at a certain flow
rate according to the USP (Table 8). The stages that had a cut-off diameter between 1 µm and 5 µm were chosen for the study.

Table 8: Aerodynamic cutoff diameters for different stages and airflow rates as per USP.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Diameter (µm) at Flow rate (L/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 L/min</td>
</tr>
<tr>
<td>1</td>
<td>14.10</td>
</tr>
<tr>
<td>2</td>
<td>8.61</td>
</tr>
<tr>
<td>3</td>
<td>5.39</td>
</tr>
<tr>
<td>4</td>
<td>3.30</td>
</tr>
<tr>
<td>5</td>
<td>2.08</td>
</tr>
<tr>
<td>6</td>
<td>1.36</td>
</tr>
<tr>
<td>7</td>
<td>0.98</td>
</tr>
</tbody>
</table>

The highlighted stages for each flow rate were those chosen for modification in the study. The same study was performed using Ham’s F-12K medium instead of deionized water. All the studies were performed in triplicate (n=3).

3.6.3. Results and Discussion

3.6.3.1. Evaporative loss at 15 L/min

The table 9 shows the loss in height of water after running air through the NGI for 10 minutes, which is the standard run time for nebulized solutions. However, the NGI was run for 15 minutes to maintain uniformity between the various studies performed. As stated previously, stages were chosen for the study based on aerodynamic cutoff diameters for each stage at a particular flow rate.
Table 9: Loss in height of water from different stages at 15 L/min after 10 minutes.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control Height loss (%)</th>
<th>Control Height loss (mm)</th>
<th>Sample Height Loss (%)</th>
<th>Sample Height Loss (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1.41 ± 0.21</td>
<td>0.21 ± 0.03</td>
<td>3.81 ± 0.11</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>1.24 ± 0.18</td>
<td>0.18 ± 0.03</td>
<td>4.02 ± 0.22</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>1.11 ± 0.02</td>
<td>0.16 ± 0.00</td>
<td>4.32 ± 0.35</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>7</td>
<td>1.25 ± 0.08</td>
<td>0.18 ± 0.01</td>
<td>4.56 ± 0.39</td>
<td>0.67 ± 0.06</td>
</tr>
</tbody>
</table>

As can be seen in the table, the loss in height of the water remains more or less similar in the control SVLIS for all the stages under study, since the control SVLIS was left outside on the countertop each time. On the other hand, the losses in height are far greater in the sample SVLIS and become higher as the SVLIS is incorporated further down in the NGI. In other words, height loss is much greater when SVLIS is at stage 7 (0.67 mm) than at stage 4 (0.56 mm).

The USP states that any difference in the critical impaction distance should be not more than 0.1 mm, which corresponds to a 0.68% loss [43]. The loss in the height of water observed here is greater than that allowed by the USP. However, the USP guidelines are for collection cups with a solid collection surface, while there is no specification for a liquid impaction surface like the one being studied here. Further, the results of the study in section 3.4.3.1 show that this change in height of water did not significantly affect the deposition or the aerodynamic parameters of the drug nebulized into the NGI containing the SVLIS at stage 4. However, the increasing change in height of water when the subsequent stages are modified (stage 5, 6 and 7) might be a cause of the significant differences observed in
the deposition patterns and/or aerodynamic parameters of drug (section 2.4.3). The size of
the orifices on the seal body decrease in size from the first to last stage of the NGI, which
makes the linear velocity (volumetric airflow divided by cross-sectional area of orifices)
of the air through the initial stages lower than that at the latter stages [62]. Thus, the higher
linear velocity in the latter stages might be responsible for the greater decrease in the water
level when the latter stages are modified due to increased evaporative loss.

The same study was performed using an equal volume of Ham’s F-12K medium instead of
water in order to determine whether it would behave the same as water when exposed to
airflow in the NGI at 15 L/min (Table 10).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (%)</td>
<td>Height loss (mm)</td>
</tr>
<tr>
<td>4</td>
<td>1.15 ± 0.05</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>1.01 ± 0.07</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>1.13 ± 0.11</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>7</td>
<td>0.93 ± 0.01</td>
<td>0.14 ± 0.00</td>
</tr>
</tbody>
</table>

For the Ham’s F-12K medium again, the change in height in the control SVLIS is almost
the same every time and much lower compared to the sample SVLIS. The loss in height of
the medium also goes on increasing as farther stages are incorporated but the difference is
not quite as pronounced as in case of water.
3.6.3.2. Evaporative loss at 30 L/min

The same experiment was also performed using deionized water to study the effect of an airflow rate of 30 L/min (Table 11), which is the standard airflow rate used to study deposition in the NGI using Metered-dose Inhalers (MDI). Even though the NGI was run for 15 minutes during the study for uniformity, only data for 1 minute is considered, since inhaler actuation takes a few seconds at most.

Table 11: Loss in height of water from different stages at 30 L/min after 1 minute.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (%)</td>
<td>Height loss (mm)</td>
</tr>
<tr>
<td>3</td>
<td>0.12 ± 0.03</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.10 ± 0.00</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.18 ± 0.06</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>6</td>
<td>0.16 ± 0.05</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

In this study, like the previous ones, the magnitude of loss in height was higher in the sample SVLIS than the control each time and it increased as progressively latter stages of the NGI were modified. However, the increase was not as distinct as the previous study. Similar results as obtained at 15 L/min were obtained in this study, but since only height loss data at the end of 1 minute was considered, the loss in height observed was not as high. The loss in height after 1 minute when the SVLIS was at stage 4 was only about 0.14 mm, which is close to the USP specification of 0.1 mm. thus, an actuation using an MDI for a span of few seconds would not markedly affect the critical impaction distance at the stage containing the SVLIS. To ensure a similar result in case water is replaced with Ham’s F-12K medium, the same study was performed using the medium (Table 12)
Table 12: Loss in height of medium from different stages at 30 L/min after 1 minute.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (%)</td>
<td>Height loss (mm)</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ± 0.1</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.4 ± 0.0</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>5</td>
<td>0.7 ± 0.3</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.6 ± 0.2</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

Similar results were observed with the medium as with water, but as discussed previously, the loss in height overall was lesser than with water and also less than or close to the USP specification of 0.1 mm.

3.6.3.3. Evaporative loss at 60 L/min

Finally, the same study was performed using deionized water by circulating airflow at a rate of 60 L/min through the NGI (Table 13), which is the standard airflow rate for experiments that use a DPI to aerosolize the drug through the NGI.

Table 13: Loss in height of water from different stages at 60 L/min after 1 minute. (Height loss of water and medium from stage 5 not shown because considerable splashing of liquid was observed at 60 L/min when SVLIS was placed in stage 5)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (%)</td>
<td>Height loss (mm)</td>
</tr>
<tr>
<td>2</td>
<td>0.27 ± 0.05</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.18 ± 0.10</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.22 ± 0.07</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Again, the experiment was performed and readings taken for a span of 15 minutes, but data considered was only up to the 1 minute mark, since DPI actuation is complete in a matter of seconds. At the end of one minute, the loss in height of water observed was again very low for the control SVLIS in each case, and higher for the sample SVLIS. The loss in height when the SVLIS was at stage 2 and stage 3 was the same (0.14 mm) while it was higher when SVLIS was incorporated at stage 4 (0.22 mm). This loss in height, and thus, change in critical impaction distance might be partially responsible for the difference in deposition obtained when deposition was studied for a DPI by incorporation of SVLIS at stage 4. An attempt was made to perform the study by keeping the SVLIS containing water in stage 5, excessive splashing of the water was observed at the first reading itself (1 minute) and the study was discontinued. Another study of the same kind was performed by replacing water with Ham’s F-12K medium to study its behavior when exposed to an airflow rate of 60 L/min in the NGI (Table 14).

Table 14: Loss in height of medium from different stages at 60 L/min after 1 minute.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (%)</td>
<td>Height loss (mm)</td>
</tr>
<tr>
<td>2</td>
<td>0.12 ± 0.03</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>0.16 ± 0.05</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.14 ± 0.07</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

In this case again it was observed that the overall loss in height of the medium was lower than that for water and each time it was around the specification in the USP (0.1 mm). An attempt was again made to perform the study by keeping the SVLIS in stage 5 but again considerable “splashing”, that is, liquid movement, was observed due to which the study
was discontinued. The splashing might be due to increased turbulence in the inter-stage passageways in the latter stages of the NGI at the high airflow rate of 60 L/min, since we have already established that the higher linear velocity in the latter stages of the NGI leads to increased evaporative losses.

In all the above studies, the loss in height of the medium was lower than that observed with water, which leads us to believe that replacing the water with medium might lead to fewer changes in the critical impaction distance. It was hypothesized that this might be due to the possibly higher density and viscosity of the medium as compared to water. Thus, density of the two liquids was determined using the Mettler Toledo DM40 Density meter (Mettler Toledo, Columbus, OH) and their viscosity was determined using the Brookfield DV-1 Digital viscometer (Brookfield Engineering Labs Inc., Stoughton, MA), which confirmed that both the density as well as viscosity of medium was slightly higher than that of water (Table 15).

<table>
<thead>
<tr>
<th></th>
<th>Density (g/cm³)</th>
<th>Viscosity (cps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>0.9982</td>
<td>3</td>
</tr>
<tr>
<td>Ham’s F-12K medium</td>
<td>1.0085</td>
<td>3.4</td>
</tr>
</tbody>
</table>

A possible reason could be elevation of boiling point of the water due to addition of the powdered medium and other solutes to it for the preparation of Ham’s F-12K medium. This is a colligative property, which depends on the solute added to the solvent, instead of the identity of the solvent itself. Thus, the added solutes in the medium may have increased its evaporation temperature, leading to a smaller loss.
The lowest change in critical impaction distance when the NGI was run at 15 L/min for 10 minutes was observed when the SVLIS was incorporated into stage 4. Also, changes in critical impaction distance that more or less matched the limit stated by the USP were also obtained when the NGI was run at 30 and 60 L/min with the modification at stage 4. The cut-off diameters for stage 4 also lie within the desirable range of 1-5 µm for all three flow rates, making it the convenient stage for modification. Keeping these factors in mind, stage 4 was chosen as the stage for incorporation of the SVLIS for all future studies.

The changes in height of water and Ham’s F-12K medium due to different airflow rates when the SVLIS is incorporated at stage 4 can be summarized in the following graphs (Figure 28).
Figure 28: Loss in height of water and medium from stage 4 at different flow rates. (a) 15 L/min; (b) 30 L/min; (c) 60 L/min
CHAPTER 4

Specific Aim 3: To study the dissolution profile of a powdered drug using the SVLIS
4. **Background**

Dissolution of an inhaled drug particle in the pulmonary fluid can be a rate-limiting step for absorption of a drug into cells, especially for drugs with low solubility and high permeability. It is proposed that the SVLIS modified NGI can be used as a model to study the dissolution of a drug in the fluid immediately after deposition in the NGI for the development of *in vitro* models that simulate the conditions a drug faces *in vivo* after inhalation.

4.1. **Dissolution study using SVLIS as the dissolution chamber**

4.1.1. **Materials**

Theophylline anhydrous was obtained from the City Chemical Corporation (New York, NY). Lactose was obtained from Sigma-Aldrich Co. (St. Louis, MO).

4.1.2. **Methods**

250.3 mg of theophylline was weighed out and homogeneously mixed with 4.7557 g of lactose and then subjected to particle size reduction using the Planetary Ball Mill PM 100 (Retsch Inc., Newtown, PA). 20.7 mg of this homogenized powder was weighed to be used as the sample for dissolution and deionized water was used as the dissolution medium for this experiment. The SVLIS which was to be used as the dissolution chamber was filled with 5 mL of deionized water and placed inside the incubator-shaker (MaxQ 4450, Thermo Scientific). The SVLIS was placed inside a petri dish before putting it in the incubator-shaker in order to keep it covered and prevent solvent loss during the experiment.
At time 0, the powder sample was added to the SVLIS and the first sample was removed. 500 µL of sample was removed from the medium at 0, 1, 2, 3, 4, 5, 10 and 15 minutes and replaced with fresh deionized water. During the experiment, the apparatus was maintained at 37ºC and at a speed of 75 rpm. The samples were then diluted 1:10 before analyzing them using the Synergy H1 Hybrid Multi-mode Microplate Reader (Biotek Instruments Inc., Winooski, VT) at a wavelength of 273 nm. The concentrations of theophylline in the samples were calculated using a standard curve which was run simultaneously in the plate reader.
4.1.3. Results and Discussion

Figure 31: In vitro dissolution profile of theophylline

The graph above (Figure 31) represents the dissolution of theophylline in deionized water over a span of 15 minutes. The total amount of theophylline at Time 0, that is, total drug content in 20.7 mg of the theophylline-lactose mixture was determined experimentally before using it to calculate the percentages of total drug recovered at each time point. The maximum dissolution of 93.65% was observed at 2 minutes ($t_{2\text{ min}}$).

Theophylline is slightly soluble in water, up to a concentration of 8.3 mg/mL. Since a very small amount of theophylline is present in the sample, dissolution is seen to be rapid and occurs within just 2 minutes. Also, theophylline was only mixed with lactose without any substantial particle engineering and formulated as an immediate release powder for experimentation. The absorption and clearance of drugs with higher aqueous solubility is not governed by their dissolution rate as much, unless the formulation is considerably altered by addition of excipients. Whereas for drugs with very poor aqueous solubility, a
rapid onset of absorption is observed followed by sustained absorption over time, which could possibly be controlled by its dissolution rate [15, 69]. The formulation would have to contain the necessary excipients and be appropriately optimized in order for dissolution time to be controlled. A low solubility drug such a steroid could also be used for dissolution testing.

For the purpose of this study, the sample was added directly to the SVLIS at time 0. If the SVLIS were to be incorporated in the NGI before performing the study, the particles would impact upon the SVLIS with greater force and at a much higher rate (at an airflow of 60 L/min). We can assume that would change the dissolution rate to a certain extent, most likely causing an increase in it and that would have to be taken under consideration during testing of any formulation using this method. This study was performed only as a proof of concept to show that the SVLIS can be potentially used for dissolution testing of orally inhaled powder formulations and that is why only an n of 1 was performed. The results obtained are evidence that the SVLIS is a potentially viable system for dissolution testing of orally inhaled drug products.

4.2. Proposed study design for future dissolution testing

If the dissolution study were to be performed after aerosolizing the drug through the NGI using a DPI, it would more closely simulate the aerodynamics of the lung during and immediately after drug inhalation. In order to be able to do that, first there is a need to achieve a deposition profile in the modified NGI containing the SVLIS that closely matches that obtained after aerosolizing a dry powder through an unmodified NGI. That might be possible by coating the collection surfaces with Sigmacote before running the NGI as explained in section 3.4.4.3 to prevent particle bounce effects.
The dissolution medium used for this study was deionized water, however for a detailed dissolution study, a medium that resembles the lung epithelial lining fluid (ELF) as closely as possible should be used. In healthy individuals, this fluid is mainly composed of mainly water (96%), salts, phospholipids, proteins and mucins with a pH of about 6.6 in healthy lung [15, 98, 99], the alveoli have a thin surface layer of lung surfactant containing phospholipids and proteins which increase the solubility of poorly soluble drugs [15, 100, 101]. Since the composition of the epithelial lining fluid (ELF) in the lungs is extremely complex, the dissolution medium to be used should preferably be a variation of simulated lung fluid (SLF), which would closely resemble the composition of the ELF in terms of electrolyte concentration [67, 102, 103]. The volume of medium to be placed in the SVLIS is low, keeping in mind the thin layer of ELF lining the lungs [15].

For this study, SVLIS was placed in the incubator-shaker to maintain the temperature at 37°C and the agitation. Another way to achieve the same would be to place the SVLIS (covered using a petri dish) on an induction plate and making use of micro-stirrer bars for agitation. The one problem in this case might be providing heat uniformly to the system since it is not enclosed as in the incubator-shaker. The speed of agitation would need to be optimized as well. The humidity in the lung is extremely high, which also needs to be considered. An alternative would be to place the system inside an incubator without any agitation at all, to simulate lung conditions [82]. Due to the small volume and surface area of the SVLIS and its open nature, sampling from the same spot every time was not possible. A small hole can be drilled on top of the petri dish so that samples can be withdrawn from approximately the same point every time.
The results obtained from further dissolution studies to be performed using SVLIS as the dissolution chamber need to be validated by comparing them with those obtained from similar studies previously performed by researchers in several different ways. A good start would be to compare results with a dissolution study performed using a conventional USP type II (paddle) apparatus. That would be instrumental in providing more information about the potential for using the SVLIS as a chamber for dissolution testing.
CHAPTER 5

Conclusions and Future studies
5. Conclusions and future directions

5.1. Conclusions

The recent advances in the area of pulmonary drug delivery and orally inhaled formulations has necessitated the development of enhanced models for *in vitro* testing, in order to enable us to better predict their behavior *in vivo*. These models must simulate as closely as possible, the deposition of the aerosol in the lung. A truly efficient model would be expected to integrate at least two aspects of aerosol delivery to the lung, for instance its deposition and subsequent dissolution or deposition followed by its dissolution in the fluid lining the lung. This Master’s project involved the design and evaluation of one such *in vitro* model.

A small volume liquid impaction surface (SVLIS) was designed as a modification for incorporation in NGI stages which would potentially be able to be used for performing *in vitro* cell studies as well as dissolution studies following inhaled drug deposition in the NGI. The SVLIS was successfully used to grow monolayers of A549 cells for incorporation into the NGI. The cells grown on the SVLIS were incorporated in the NGI at stage 4 before exposing them to airflow and subsequently saline at 15 L/min inside the cell culture hood. Even though airflow alone was shown to somewhat inhibit the growth of cells, saline when administered to the cells did not adversely affect their growth. Thus, the SVLIS can be said to have a potential application in *in vitro* cell culture models for studying absorption of inhaled drugs following deposition through an impactor.

Even though cell cultures could be incorporated in the SVLIS modified NGI, validation studies showed that deposition patterns of the model drug theophylline were affected following the modification at all stages (5, 6 and 7) except stage 4. This was partially
attributed to an excessive loss in height of the liquid in the SVLIS that occurs when exposed to airflow, thus affecting the critical impaction distance. Hence, it was recommended that stage 4 be modified for all further studies and airflow dynamics in the NGI be evaluated in detail before other stages could be modified. Deposition of dry powder obtained at modified stage 4 were also observed to be different from those of unmodified NGI, which could be possibly attributed to the use of uncoated collection surfaces.

A preliminary dissolution study of a model drug using the SVLIS as a dissolution chamber suggested that the SVLIS can be potentially used in an *in vitro* dissolution model to study inhaled drug dissolution after deposition, provided an appropriate method was designed by optimization of all the parameters. However, the problems encountered in the deposition patterns obtained after dry powder aerosolization need to be resolved before further dissolution studies can be undertaken.

Thus, we can conclude that the SVLIS can be potentially incorporated at stage 4 of the NGI for further *in vitro* cell studies following deposition of nebulized drug solutions. However, further studies need to be performed as described in the future studies section before its application for modification of other stages or for dissolution studies of inhaled dry powders can be established.

### 5.2. Future studies

Deposition profiles obtained following nebulization of theophylline in modified NGI with SVLIS at stages 5, 6 and 7 were found to be different from those obtained from the same study using unmodified NGI. Thus, further studies need to be performed in order to examine the deposition patterns and airflow dynamics inside the NGI to determine if any
changes in airflow rate and turbulence were occurring towards the latter NGI stages which were responsible for the variations in deposition patterns in the SVLIS modified NGI. Also, the validation studies using dry powder inhalers should be performed again after coating the NGI collection surfaces with the appropriate coating solution to reduce particle rebound and re-entrainment, in order to verify the utility of this model to study dry powder deposition.

The utility of this model in studying drug absorption and transport should be further tested by performing cellular uptake studies following deposition of a model drug solution on the cells grown on the SVLIS and by performing transepithelial electrical studies (TEER) studies across the cell monolayers, in order to actually study the effect of the environment in the NGI on the pharmacokinetics of a known drug. An attempt should be made to grow other lung epithelial cell lines such as Calu-3 to verify that this system can be applied across a range of cell lines representing different regions of the lung epithelium.

Dissolution studies should be appropriately designed by alteration and optimization of parameters like agitation, humidity, sampling and using a proper inhaled formulation. Dissolution testing of a commercially available inhaled product such as a steroid should be performed to further establish the validity of this model in in vitro dissolution testing.
References


