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Review

In vitro, *in vivo* and *ex vivo* models for studying particle deposition and drug absorption of inhaled pharmaceuticals

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ABSTRACT

Delivery of therapeutic agents via the pulmonary route has gained significant attention over the past few decades because this route of administration offers multiple advantages over traditional routes that include localized action, non-invasive nature and favorable lung-to-plasma ratio. However, assessment of post administration behavior of inhaled pharmaceuticals-such as deposition of particles over the respiratory airways, interaction with the respiratory fluid and movement across the air-blood barrier-is challenging because the lung is a very complex organs that is composed of airways with thousands of bifurcations with variable diameters. Thus, much effort has been put forward to develop models that mimic human lungs and allow evaluation of various pharmaceutical and physiological factors that influence the deposition and absorption profiles of inhaled formulations. In this review, we sought to discuss *in vitro*, *in vivo* and *ex vivo* models that have been extensively used to study the behaviors of airborne particles in the lungs and determine the absorption of drugs after pulmonary administration. We have provided a summary of lung cast models, cascade impactors, noninvasive imaging, intact animals, cell culture and isolated perfused lung models as tools to evaluate the distribution and absorption of inhaled particles. We have also outlined the limitations of currently used models and proposed future studies to enhance the reproducibility of these models.

Contents

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1. Introduction

The human lung is one of most complex organ systems of the body that are composed of 300–500 million alveoli and a surface area of ≈100 m^2. The main functions of the lungs are to transport oxygen from the environment into the blood and move carbon dioxide back to the atmosphere. In addition to being the conduit for supply of oxygen and removal of carbon dioxide, lungs also serve as the port of entry for air borne particles that include environmental pollutants, medicinal and gaseous substances. For this reason, lungs have long been used for inhalation of recreational substances. In modern medicine, use of lungs as a route of administration has been limited mainly to the delivery of medication for obstructive pulmonary diseases such as asthma, bronchitis and emphysema (Gonda, 2000; Sobande and Kercsmar, 2008). In fact, most of the currently available inhalation products are used for the treatment of diseases that are localized in the lungs (Siekmeier and Scheuch, 2008; Sobande and Kercsmar, 2008). In addition to treating diseases of airways, lungs have also been used as a route for targeted therapy of various disorders of the lungs that include cystic fibrosis, pulmonary vascular disorders such as pulmonary arterial hypertension (Gupta et al., 2011a), interstitial lung disorders and non-small cell lung cancer (Gettinger, 2008; Haj et al., 2006; Ramalingam and Belani, 2007). Furthermore, over the past several decades, lungs have been extensively investigated as a route for administration of therapeutic agents that produce systemic effects instead of localized actions in the lungs. Indeed, we and others have studied the feasibility of lungs as a site of administration of a number of drugs, including insulin (Al-Qadi et al., 2012; Bi et al., 2009; Hamishehkar et al., 2010; Lim et al., 2009; Martin et al., 2008; Patel et al., 2009; Perring et al., 1994; Yang et al., 2012; Zhao et al., 2012), low molecular weight heparin (Bai and Ahsan, 2009, 2010; Bai et al., 2010; Patel et al., 2012a; Yang et al., 2006; Yang et al., 2004), hepatitis B vaccine (Chengalvala et al., 1991; Lombry et al., 2004; Thomas et al., 2008), that are systematically effective. The physiological and anatomical factors that propelled the lungs to be used as the systemic route of administration are enormous absorptive surface area (>100 m^2) (Huang and Wang, 2006; Patton et al., 2004b) and extremely thin single cell layer that separates the alveolar epithelium from the dense vasculature and forms a highly permeable membrane (Liu et al., 1993). Lungs have also been used as alternatives to the parenteral route of administration because of the possibility of needle-free treatment, ease of self-administration and avoidance of injection related complications. Inhaled drugs can bypass the enzymatic degradation that occurs upon oral administration and thus allow the drug to be administered at a reduced dose. Compared to orally administered drugs, inhaled drugs are available in the systemic circulation at a faster rate and can avoid limitations imposed by food intake (Patton et al., 2004a; Yu and Chien, 1997).

Thus, inhalable drug delivery presents a viable alternative to the traditional drug delivery system. However, due to the complexity of the respiratory system, it is practically impossible to understand various aspects of drug deposition patterns and absorption via the lungs. To understand the influence of various physiological and pharmaceutical factors on the deposition patterns and absorption profiles of inhaled drug formulations, a series of *in vivo*, *in vitro* and *ex vivo* models mimicking one or more anatomical and physiological aspects of human respiratory system have been proposed and studied extensively (Cypel and Keshavjee, 2012; Martonen et al., 2005; Sakagami, 2006; Villenave et al., 2012). However, none of the currently used models adequately reproduces various features of the respiratory apparatus. Therefore, it is important to identify the limitations of existing models and propose novel strategies to address those limitations.

In this review, we have discussed various *in vitro*, *ex vivo and in vivo* models that have been extensively used to study particle deposition patterns and absorption profiles of therapeutic agents following pulmonary delivery. The manuscript is divided into two main sections that discuss the models for studying the deposition and absorption of inhaled pharmaceuticals.

2. Models for studying deposition patterns of inhaled therapeutics

For therapeutic efficacy, inhaled drug formulations must efficiently deposit in the airways and be available to produce therapeutic action locally or efficiently traverse the air-blood barrier to produce systemic effects. Deposition generally refers to the mean probability of a particle being deposited in the respiratory tract upon settling on airway surfaces. The deposition of inhaled particles in the respiratory airways is strongly influenced by a number of parameters that include mass median aerodynamic diameters (MMAD), density and shape of the particles and various physiological and anatomical factors of the respiratory system. Indeed, there is a direct relation between particle distribution and the MMAD (Ferron, 1994), which is defined as the diameter of the aerosol particles that divides the mass distribution into half (Labiris and Dolovich, 2003). In addition to geometric particle size, shape, density and hygroscopicity of particles also play roles in the deposition patterns of inhaled particles. A widely accepted notion is that for efficient deposition, MMAD should be in the range of 1 and 5 μm (Byron et al., 2010; Shi et al., 2009). Smaller sized particles are likely to be exhaled or absorbed quickly from the airways which may pose a risk of systemic toxicity. This aspect is sometimes advantageous as is observed in the case of cystic fibrosis where particles must reach the bronchioles (Ferron, 1994). In contrast, larger particles are cleared out by the mucociliary clearance mechanism (Stahlhofen et al., 1990). Some investigators suggest that particles with size 50–100 nm deposit in the alveoli whereas particles of 1–5 μm are deposited in the lower airways (Effros and Mason, 1983; O'Callaghan and Barry, 1997). Recent studies suggest that particles with a density less than 0.4 g/ml and geometric diameter greater than 5 μm also favor efficient deposition in the lungs (Edwards et al., 1997; Yoo et al., 2011).

Various anatomical, physiological and pathological conditions also influence the deposition and absorption of inhaled pharmaceuticals. In fact, airway geometry is one of the most important parameters in determining the deposition patterns of particles in the lungs. Lung airways are highly branched, the path gets narrower with increasing generations (Fig. 1) and that the air velocity varies in various zones of the respiratory apparatus (De Marzo et al., 1989). As the airway radius becomes smaller, the probability of particle deposition over any specific area also increases. Moreover, the humidity in the lungs is relatively high which may also affect the particle size and their deposition patterns in the airways. Aerosolized droplets may undergo changes in their size and distribution patterns depending on the water content of various areas of the lungs (Phipps et al., 1994). There are two methods by which influence of humidity on particle size can be studied.

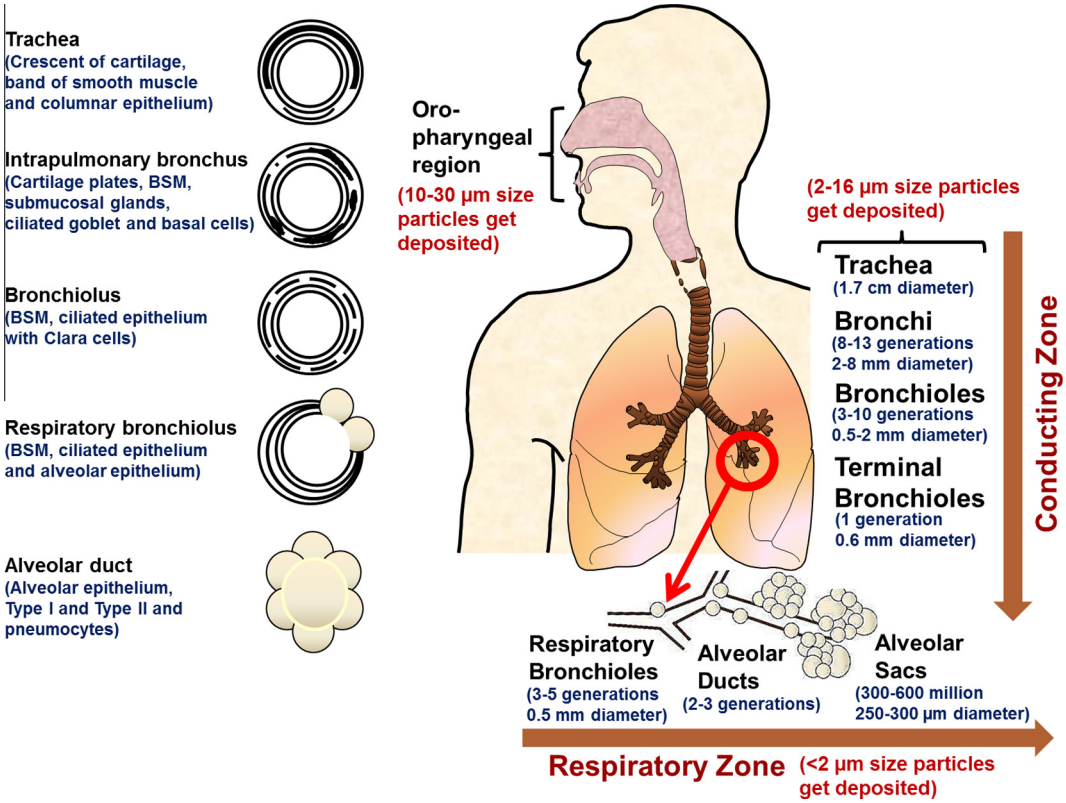

Fig. 1. The number and dimensions of the airways of the adult lung and structure of the airway wall with the generations as explained by Weibel's tracheo-bronchial tree.

One method is called enhanced condensational growth (ECG) method, in which the submicrometer aerosol is inhaled with water vapor saturated warm air that aids increase in particle size (Longest et al., 2010) and the second method is excipient enhanced growth (EEG) wherein submicrometer particles are formed at first that contains both active agents and hygroscopic excipients (Hindle and Longest, 2012; Longest and Hindle, 2011; Longest et al., 2012). Tidal volume and flow rates have also been shown to influence the deposition. As the tidal volume increases, total particle deposition also increases (Bennett et al., 1999). There are also many conflicting assumptions as to how gender influence particle deposition, although Kim and Jaques showed that women show higher deposition than men (Jaques and Kim, 2000).

Inflammation, congestion and constriction of the airways can also affect the absorption and deposition patterns of particles. Obstruction in the airways basically occurs due to accumulation of unwanted materials in the airway lumen which is supported by mucus accumulation, increased muscle tone, edema and presence of inflammatory and epithelial cells. This obstruction leads to decrease in surface area of the lungs and consequently cause reduced absorption and increased deposition of particles in the central airways. The airway obstruction is assessed by a widely used parameter "Forced Expired Volume at 1 s (FEV_1)" which decreases with increased obstruction (Labiris and Dolovich, 2003). Physiological clearance mechanisms of the lungs also dictate particle deposition patterns. Large particles, deposited in the conducting airways, are cleared by ciliated epithelia and transported further towards the pharynx and then to the gastrointestinal tract (Messina et al., 1991). In some diseased state such as cystic fibrosis, primary ciliary dyskinesia (Livraghi and Randell, 2007; Noone et al., 1999), ciliary functions are impaired and thus the clearance is adversely affected.

Thus, it is important to study the patterns of deposition of particulate formulations upon pulmonary administration. In a recently published review, we have discussed *in silico* and bioengineered lung models that can be utilized to study particle deposition pattern (Patel et al., 2012a). However, because of a higher degree of complexity and the lack of expertise required for those models, several other simpler models are being extensively used with various levels of accuracy and reproducibility. Below we have discussed some of the models that attempted to simulate the lung airway structure and have routinely been used to determine the particle deposition patterns in laboratory setup.

2.1. Lung cast models to study particle deposition

Lung cast models have long been used to study various characteristics of the lungs. Both solid negative and hollow positive casts have been used to study various aspects of the respiratory apparatus. Solid negative casts, representing lumen of the tract, are employed to study the size, anatomical features of the lungs, anatomical direction of the lung airways and adjacent vasculatures. Hollow positive casts that represent wall of the tube, on the other hand, were utilized to study fluid mechanics and deposition patterns of inhaled particles. Lung cast models of human, rat, hamster, monkey and dog have been developed using various materials including silastic rubber, resin, metal, alloy and wax (Patra and Afify, 1983; Yamada et al., 1998; Yeh et al., 1979).

Solid human lung cast was used as the base while rigid synthetic rubber resin was used to make the hollow central airways by molding around the human lung. In this model, irregularly divided branching of the tube network of the hollow cast symbolizes the real geometry of the central airways of the human lungs with relative accuracy. The purpose of this model was to analyze the relationship between steady pressure flow of the cast and the five generations of the tracheobronchial tree, and to show the influence of the larynx on the pressure-flow in the airways (Ben Jebria et al., 1987). Lung cast models, prepared from actual human lung, were

used to study the velocity profiles in the region of the carina in mechanical lungs (Patra and Afify, 1983). The model was capable of simulating breathing cycles and measuring the velocity of light, medium and deep breathing. Human lung casts can also be prepared with styrene polymer and are suitable for three dimensional analyses by simple microscopy and scanning electron microscopy. These models can also be used in computerized measurement of bronchioles, pulmonary arteries, capillaries, alveoli, tissue remnants and inhaled particle depositions in the lungs of different animals (Hojo, 1993). Yamada et al. developed a human respiratory tract cast for studying aerosol deposition in the respiratory tract. These authors employed a stereo lithographic method that used a photo curable resin and a three dimensional CAD (computer Aided Design) from the anatomical data (Yamada et al., 1998). In addition, a solid rat lung cast model representing the entire and a portion of the lung has been reported. This model is easy to handle and allows calculation of particle deposition in various parts of the lungs (Yeh et al., 1979).

2.2. Cascade impactors to determine particle deposition

Cascade impactors (CIs), including multi-stage liquid impinger (MSLIs), are the most widely used instruments to measure the size and the deposition patterns of particulate formulations delivered via the pulmonary route (Mitchell and Nagel, 2003a). However, CIs should not be considered as *in vitro* simulators of lungs, chiefly due to the discrepancy in the flow rate when compared with that of a functional lung. It is important to keep in mind that a CI operates at constant flow rate but human lungs have a varying flow rate originating from the breathing cycle. Nevertheless, CIs can provide useful information regarding particle size on the basis of aerodynamic diameter and can predict the deposition patterns of particulate drug carriers in the respiratory tract. A number of CIs are available to evaluate aerosol characteristics and the most frequently used ones are listed in Table 1.

Andersen cascade impactor (Fig. 2A) is one of the most frequently used impactors to test inhaled products. It consists of 8 stages (Mohammed et al., 2012) that are constructed in a pattern such that larger particles having sufficient inertia will impact upon particular stage collection plate as the aerosol stream passes through, whereas relatively smaller particles with insufficient inertia will be carried in the air stream and pass to the next impaction stage (Mitchell et al., 2010). Hence, it is feasible to determine the fine particle dose (FPD) (Weda et al., 2004), fine particle fraction (FPF) (Martin et al., 2006) and subsequently mass median aerodynamic distribution (MMAD) by quantifying the amount of drug particles deposited on each stage (Gupta and Ahsan, 2011). In contrast, the Marple-Miller Impactor 160 (Fig. 2B), which is described as Apparatus 2 in the United States Pharmacopeia (USP), has five stages and can also be used to determine aerodynamic diameters of particles (Nasr et al., 1997). This instrument is equipped with removable collection cups that help quick and simple recovery of drug particles without dismantling the device (Feddah et al., 2000). A multi stage liquid impinger (MSLI) (Fig. 2C) is also a five-stage system that can be used to determine aerodynamic diameters (Sebti and Amighi, 2006). The collection stages of MSLI are kept moist to attenuate possible re-entrainment that can occur with ACI or MMI (Taki et al., 2010). Further, the next generation impactor (NGI) was developed in 2000 by a combined effort of a group of pharmaceutical companies. It is a high performance, particle classifying cascade impactor comprising seven stages with a micro-orifice collector (Marple et al., 2003). The flexibility and high productivity have made NGIs a popular CI for industrial application.

Theoretical parameters, which serve as basis for choosing impactors, are equivalent particle diameters and cut-point of impactors. Drugs given via the inhalational route not only differ in their size distribution but also their physical state, density, shape and velocity (Shekunov et al., 2007). These factors influence their deposition in the respiratory tract. Several forces, such as inertial force, gravity and inspiratory air, act on the particles travelling through the tract. Impactors generally depend on the counterbalance of two opposing forces which are drag and centrifugal forces (de Boer et al., 2002). Cut-points of inertial impactors are represented as particle sizes with a collection efficiency of 50% (Dunbar and Mitchell, 2005). Stoke number is used to derive the collection efficiency which is the ratio of particle stopping distance to the impactor nozzle distance. Particle bouncing is usually prevented by coated plates. Effect of temperature, pressure difference and humidity on the inhaler and airflow rate is optimized before testing (de Boer et al., 2002). Some important features of inertial impactors include deposition by inertial impaction mechanism, fixed dimensions of the conducting tubes, well regulated inspiratory flow curve, efficient particle collection, and bent curvature of the inlet tube.

There is very limited information regarding comparative efficiencies of various CIs used to study particle deposition patterns. Olsson et al. reported that the size distribution of budesonide turbuhaler DPI was consistently narrower in ACI compared to those obtained with the MSLI (Olsson, 1995). However, MMADs measured by both CIs were within a similar range having ACI-based and MSLI values at the upper and lower end, respectively. It is also reported that the size distribution from pMDI-delivered peptide suspension formulation resulted in a significantly larger value of MMAD by MMI compared to that by ACI (Rao et al., 1997). The authors attributed this difference to the higher inter-stage losses of larger particles with ACI, leading to over-estimated proportion of finer particles. Some non-aerodynamic methods, such as laser diffractometry, laser doppler particle size analysis, single particle light scattering and microscopy-automated image analysis, have also been used to characterize particle deposition (Mitchell et al., 2011). However, these above mentioned new techniques are still in their infancy and need more optimization for more efficient use.

It is important to note that use of cascade impactors in determining aerodynamic diameter and deposition patterns of particles is challenged by 'particle bounce effects', particularly when DPI based formulations are used. Hence, both US and European Pharmacopeia emphasize on coating the plates with silicone oil to reduce particle bounce and inter-stage loss that could be critical towards successful characterization of aerodynamic distribution of inhalable particles (Wong et al., 2010). As bounce effect is reported to be drug and dose specific, an optimization should be

Table 1
Cascade Impactors used to study particle deposition pattern.

Cascade impactors	US pharmacopeia	European pharmacopeia
Andersen 8-stage (ACI) – no pre-separator	Apparatus 1 for pMDIs	Apparatus D
Marple-Miller Series 160 (MMI)	Apparatus 2 for DPIs	–
Andersen 8-stage (ACI) – pre-separator	Apparatus 3 for DPIs	Apparatus C
Multi stage liquid impringer	Apparatus 4 for DPIs	Apparatus C
Next generation pharmaceutical impactor (NGI)	Apparatus 5 for DPIs	Apparatus E

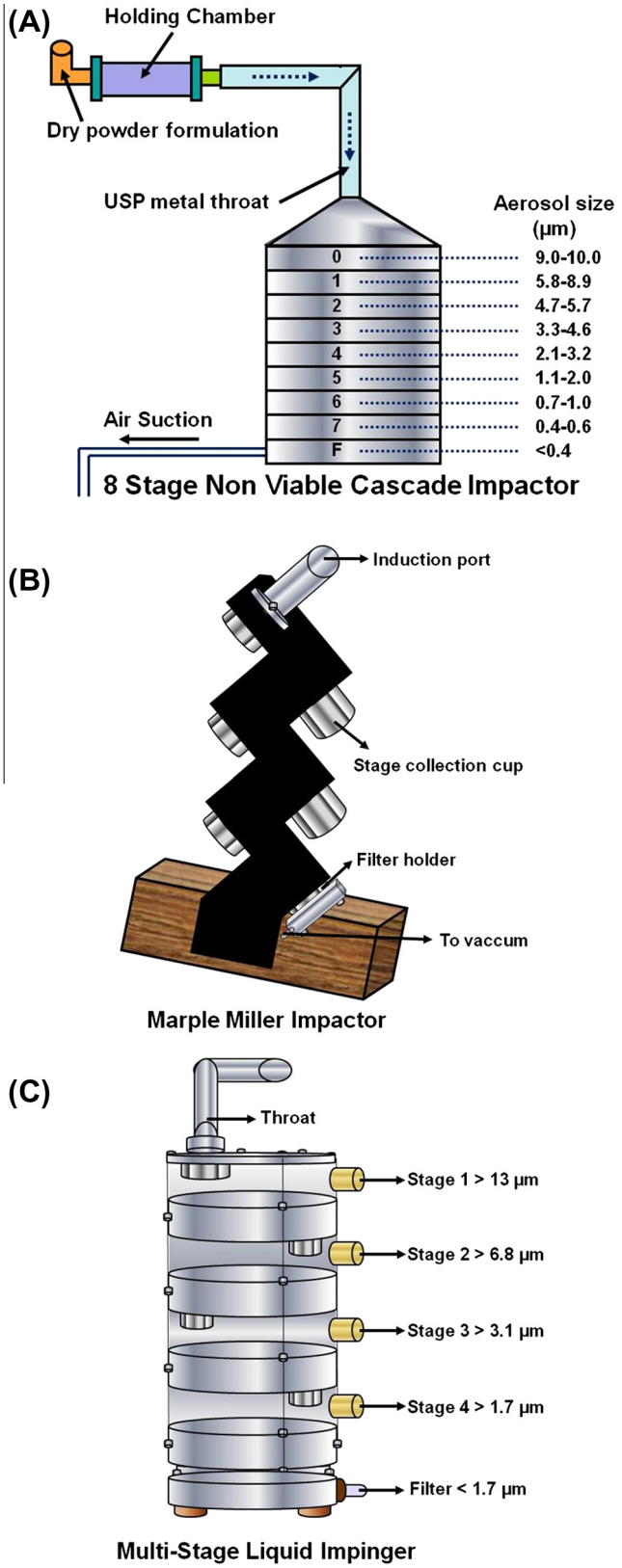

Fig. 2. Schematic diagrams of (A) Andersen cascade impactor, (B) Marple miller impactor and (C) Multi-stage liquid impinger.

carried to determine the cumulative dose up to which such event can be avoided (Hindle, M., Int J Pharm, 1996 (134)). However, it has been demonstrated that even a very low dose pMDI, such as 100 µg albuterol, can end up with varying deposition profile between Marple Miller or ACI impactor if the plates are not coated (Nasr et al., 1997). It has also reported that jet velocity, for example 60 L/min with 5–10 mg large porous particle to an ACI, can produce bounce effect, which can be reduced, but not eliminated, by reducing the velocity (Dunbar et al., 2005). Furthermore, when NGI and ACI were compared with high dose (5 mg zanamivir) and high velocity 90 L/min), the former with coating was found to be within pharmacopeial guideline for impactor losses (<5%) (Kamiya et al., 2009). Thus, for a particular drug and/or formulation, the dose, jet velocity and type of impactor should be optimized to minimize particle loss and hence, and to obtain meaningful outcomes.

2.3. Particle deposition in live animals

Various tools such as twin-stage impinger, multi-stage liquid impinger and cascade impactors can categorize particle based on their size which are useful in predicting *in vivo* deposition of particles, and hence are important tools in the development and quality control of new pharmaceutical products. However, these methods have some limitations as they do not adequately mimic the upper and lower respiratory tracts (Newman and Chan, 2008). An approach to resolve this problem could be capturing images of particulate drug carriers *in vivo* by means of various methodologies such as gamma scintigraphy (Newman, 1993), single photon emission computed tomography (SPECT) (Snell and Ganderton, 1999), positron emission tomography (PET) (Gardner et al., 1992), magnetic resonance Image (MRI) (Thompson and Finlay, 2012) and fluorescence imaging (Yi et al., 2012). Currently used *in vivo* imaging technique can measure total lung deposition and oropharyngeal deposition of particles directly by using radionuclides (Gardner et al., 1992), nonionizable radiation (Thompson and Finlay, 2012) and fluorescent dye (Yi et al., 2010). Deposition patterns can be examined directly by taking images of lungs or by autoradiography after sacrificing and dissection of animal lungs. However, before using these techniques, methods to be pre-validated and minimal exposure of the radiation to body tissues need to be ensured. Better precision of data can be obtained from techniques such as SPECT and PET which produces 3D images compared to 2D image producing technique, gamma scintigraphy. 3D images allow more detailed data on regional lung deposition (Snell and Ganderton, 1999). However, *in vivo* methods that use isotopes have some drawbacks including high cost, higher radiation doses, safety hazards, and specialized training required in handling of radio labeled isotopes. Of all the techniques, magnetic resonance imaging (MRI) and fluorescent imaging are considered to be the safest. Overall, the use of *in vivo* imaging techniques is beneficial over *in vitro* techniques as these techniques can provide an actual picture of regional deposition of particles in the lungs. These techniques can be used to evaluate both qualitative and quantitative aspects of particle deposition in the lungs and can be a useful means to understand the relation between deposition of drugs in the lungs and their clinical effects. In this section, we have briefly discussed the basic features, advantages and shortcomings of these techniques for measurement of regional deposition of particles in live animals. We also made recommendations for identifying an acceptable technique and suggested issues that need to be addressed in imaging based systems used to evaluate deposition patterns of inhaled particles.

2.3.1. Gamma scintigraphy

Gamma scintigraphy, a widely used method that measures particle deposition and mucociliary clearance studies in lungs. In this method formulations are labeled with gamma ray emitting radioisotopes (Newman, 1993). Prior to performing this measurement, radiolabelling process should be properly validated, and background radiation, image duration, depth of the source and

reduction in gamma exposure to body should be checked. This method also allows studying regional lung deposition by taking 'regions of interest' in a scintigraphic image. Following inhalation of the formulations, thorax is scanned by a gamma camera, and the radioactive counts can then be digitized to get a 2D image of the oropharynx, lungs and stomach along with inhalational device and exhalation filter. In this method, the outer side of the lungs can be imaged by ^{81m}Kr ventilation scan or a transmission scan, and be superimposed to quantify penetration and distribution of the gamma-labeled particles. Additionally, when drugs are not labeled with radioisotopes, they can be incorporated in formulations, in which radioisotopes will be distributed inside the particles in an equal fashion. However, since this method takes 2D images, it allows overlay of the structures of interest with a possible failure to distinguish between alveoli, small and large airways that are all positioned centrally. Penetration index (PI), which is the ratio of peripheral to central deposition, reveals the degree to which the aerosol reaches the smaller airways in the lung periphery. From nebulized formulations, this index can estimate the correlation with the split between tracheobronchial and alveolar deposition (Newman et al., 2001).

2.3.2. Single photon emission computed tomography (SPECT)

In SPECT, radiolabeled formulations are used to take planer images. The gamma camera, instead of taking interior and posterior picture of the thorax, rotates around the subject at 360 °C (Fleming et al., 2011) and gives topographic picture of coronal, sagittal and transverse planes. Computer systems then process these pictures and convert them to 3D construction like X-ray computed tomography (CT) (Snell and Ganderton, 1999). Thoracic CT scan confers better anatomical localization of the radionuclide, aids in mapping attenuation of gamma emission by the thoracic structure, and hence enhances the accuracy of the method (Perring et al., 1994). However, since this method takes 30 min to complete *in vivo* measurement of regional deposition, redistribution of radioaerosol by mucociliary clearance, coughing and absorption into blood stream may occur within the experimental duration (Huchon et al., 1987). Additionally, the application of this method is sometimes challenged by the requirement of a high dose. Recently, MRI is being used for the same purpose because of the advantage that it does not use additional radiation dose. However, higher cost and greater technical requirements limit its effectiveness as compared to planer imaging (Everard, 1994).

2.3.3. Positron emission tomography (PET)

PET method involves labeling of drugs with positron emitting radioisotopes (Cherry and Gambhir, 2001; Dolovich, 2009; Phelps, 2000). The labeled positrons produce two high-energy photons by annihilating with electrons, which are emitted simultaneously at 180° to each other, detected, counted and recorded as a single event. To get accurate delineation of regions of interest, the resulting 3D image can be co-registered with an MRI or spiral CT scan of the thorax which eventually gives the percentage of the inhaled drugs dose administered to these regions. This method has a major advantage as it allows the drug itself to act as radioactive tracer by incorporating some positron emitting isotopes e.g. ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{64}Cu and ^{124}I in non-pharmacological trace quantities (Park et al., 2012) that can be directly included to the drug by isotopic substitution (Carvalho et al., 2011; Conway, 2012; Gardner et al., 1992). PET can be used to study pulmonary pathology, biochemistry, inflammation, transgene expression and cellular response *in vivo* (Park et al., 2012; Piwnica-Worms et al., 2004) However, the limited availability of positron emitting radionuclides, and the need for the study site to be located close to cyclotron because of short half-lives (half-life of ^{11}C is 20 min) challenge the applicability of this method (Saha et al., 1992). Moreover, neither PET nor SPECT offer advantages over planner gamma scintigraphy and conventional pharmacokinetic studies for the quantification of total lung deposition of inhaled medication (Snell and Ganderton, 1999).

2.3.4. Magnetic resonance Imaging (MRI)

Since above described techniques, such as SPECT and PET (positron emission tomography), involves radiolabeling of aerosol formulations, their application is often limited by safety and lack of technical expertise to carry out the experiments. The problem of using radionuclides can be overcome by using MRI (Thompson and Finlay, 2012). Previously most MRI experiments were used to determine qualitative deposition of particles in animals (Martin et al., 2008). Recently, MRI has been used for quantification of regional deposition of aerosol in animals (Martin et al., 2008; Sood et al., 2008; Sood et al., 2010) and *in vitro* human airways replicas (Thompson and Finlay, 2012). The principle is based on Nuclear Magnetic Resonance (NMR) phenomenon that uses nonionizable radiation, soft tissue contrast mechanisms and arbitrary orientations for 2D and 3D imaging. Images are generated from the signals acquired from nuclei with either full or half integer values of spin proportional to the nuclei's angular momentum, which is a result of an odd number of protons and/or neutrons, e.g. protons found in ^{1}H, ^{3}He, or ^{15}Na. Most of the MRIs are based on the hydrogen atom found in tissues. However, in case of lung, images using helium based MRIs are now well established. MRI experiments work through interactions of nuclear magnetic moments, intrinsic to the sample or tissue of interest, with external magnetic and electromagnetic fields. MRI creates images based on sensitivity to spin density, T_1 and T_2 relaxation time and motion which ranges from diffusion to rapid pulsatile blood flow in major arteries. Here, T_1 and T_2 are the longitudinal relaxation time constant and transverse relaxation constant, respectively. These two constants give different values depending on the local environment of the nuclei that may be different for healthy tissues and pathologies (Thompson and Finlay, 2012). In order to image ventilation and aerosol delivery, hyperpolarized gas MRI that uses hyperpolarized ^{3}He or ^{129}Xe (Mosbah et al., 2008) are better than water proton imaging. These hyperpolarized gases produce higher level of polarization (Thompson and Finlay, 2012). Helium stays in the lung airspace as this gas is largely non-lipid soluble. On the other hand, the lipid solubility and affinity for biological molecules consider Xenon to be a potential biosensor (Taratula and Dmochowski, 2010). ^{3}He MRI can also be used to provide image of inhaled super paramagnetic iron oxide particles by their perturbation of the static magnetic field, not by reduction in T_1 and T_2 relaxation time. By changing the static field, it is possible to measure the amount and distribution of iron particles in the airways (de Rochefort et al., 2010). Recent research has shown that MRI can also be used for targeted delivery of particles loaded with iron oxide nanoparticles by increasing the concentration of particle deposition by placing an external magnetic field (Richard et al., 2004).

2.3.5. Fluorescence imaging

To avoid the complexities associated with radiolabeled isotopes, a fluorescence method can also be employed wherein, unlike radio-labeling, particles are fluorescently tagged and hence can be utilized to monitor deposition patterns in live animals (Ntziachristos et al., 2008). However, this method has also some problems as both the excited and emitted fluorescent lights are scattered, reflected, and absorbed by tissue, that affect the relationship between light captured by the detector and number of molecules. Biological tissue, which has the capability to auto-fluoresce, gives rise to heterogeneous background signal (Yi et al., 2010). This problem can be solved by subtracting auto-fluorescence with blank tissue or current instrumentation that emits light at long wavelengths in the near-infrared region of the spectrum (Adams et al.,

2007; Comsa et al., 2008). Fluorescent dyes such as aluminum (III) phthalocyanine chloride tetrasulfonic acid, can be administered in aerosol form and then image can be taken by *in vivo* imaging system to visualize particle deposition in different lobes of the lungs followed by quantification of particles using image analyzer. By this method, it is possible to reduce tissue attenuation by using molecular probes that absorb and emit light in the near infrared region (Adams et al., 2007; Comsa et al., 2008; Kovar et al., 2007).

3. Models to study drug absorption following pulmonary administration

The absorption profiles of drugs administered via the pulmonary route are evaluated for both locally and systematically acting drugs. For locally acting drugs, pulmonary absorption profiles are determined to assess the amount of drug that is likely to enter the systemic circulation (Chen et al., 2012; Gupta et al., 2011b; Hamishehkar et al., 2010). Pulmonary absorption profiles also give information regarding the amount of drug that would be available locally to produce therapeutic effect in the lungs and overall systemic exposure of the drug (Patton et al., 2004b). However, pulmonary absorption studies of systemically active drugs are performed to determine the bioavailability (Siekmeier and Scheuch, 2009; Tronde et al., 2003) and biodistribution of inhaled formulations (Xie et al., 2010). The data on the absorption and transport of inhaled formulations across the lungs also provides information concerning the metabolism of drugs in the lungs (Dugas et al., 2013). Although there is no direct correlation between the absorption of inhaled drugs from human lungs and that of small laboratory animal lungs, a number of *in vitro*, ex vivo and *in vivo* models have long been used to predict absorption and transport of inhaled drugs in humans, which are summarized below.

3.1. In vivo intact animal models

In vivo intact animal models are mainly used for studying the absorption, distribution and pharmacodynamics of inhaled pharmaceuticals. In intact animal models, anesthetized or conscious animals receive formulations by means of various delivery devices with or without surgical intervention. Both large and small animals have been used to study the pharmacokinetics and pharmacodynamics of various inhaled therapeutic agents (Enna and Schanker, 1972b). Of the small animals, mice and rats have been extensively used for studying the pulmonary pharmacokinetics of both large and small molecular weight drugs. Use of larger animals such as guinea pigs, rabbits, dogs, sheep and monkeys have also been reported (Cryan et al., 2007), although their use has been very limited because of high cost and logistics required for housing and handling of larger animals. Use of rats in studying the pulmonary absorption of low molecular weight drugs can be traced back to early 1970's to 80's. In a series of studies, Schanker and Colleagues first showed that it is feasible to study absorption profiles of small molecules (Enna and Schanker, 1972c) upon intratracheal administration to rats (Burton and Schanker, 1974; Enna and Schanker, 1972a; Schanker, 1978). In this earlier method, drug was administered to the lungs using an intratracheal tube after surgical exposure of the trachea. However, their proposed method was terminal and required destructive tissue sampling for each time point which was considered as major limitations for studying pharmacokinetics in intact animals. Later this method was modified and improved to eliminate the requirement of surgical intervention and avoid sacrificing animals for each time points (Ahsan et al., 2001; Thomas et al., 2008). Recently, this method has largely been replaced by a non-invasive method that uses aerosolizers for small animals (Fig. 3). Using small animal aerosolizers or insufflators developed by PennCentury (Philadelphia, PA), it is now possible to noninvasively administer both liquid and dry powder formulations to the lungs of both mice and rats. We and others have shown that both devices can reproducibly deliver a given dose to the lungs of anesthetized rats (Patel et al., 2012b; Sung et al., 2009a). In a number of papers, this method has been described in detail (Cryan et al., 2007; Qi et al., 2004). For administration of drugs using these devices, the trachea of anesthetized animals is visualized by using a small animal laryngoscope for inserting Penn-Century® Liquid Microsprayer® or dry powder insufflator inside the trachea. Drug is spray-instilled into the lungs by pushing the syringe plunger of the devices. These devices can deliver liquid or powder in the form of aerosolized droplets or particles with a mass median diameter of ~16–22 μm (Suarez et al., 2001). Intratracheal administration with positive pressure ventilation and automated nebulization has also been used to maximize deep lung deposition (Niven, 1995; Oberdorster et al., 1997). In this method, blood sampling is performed by means of surgical catheterization or tail vein milking. Sampling without sacrificing animals allows collection of blood samples from same animal and thereby eliminates inter-animal variability and reduces the total number of animals used in a given experiment. This method has various advantages, such as both small and relatively large dose of drugs can be administered in the lung and with the availability of highly sensitive method of quantitation, it is now possible to determine a vanishingly small amount of drug in lung tissue and blood (Cryan et al., 2007). In addition to pharmacokinetic studies, *in vivo* animal methods have also been used to study the distribution of drug formulations in various lung regions (Yi et al., 2010). However, noninvasive intratracheal administration suffers from a number of limitations. This method does not reflect normal physiological condition because drugs are required to be administered to anesthetized animals. Moreover, as this method uses anesthetized animals, multiple dosing is not recommended and repeated insertion of inhalation device may cause injury in the trachea (Cryan et al., 2007). In fact, the requirement of anesthesia during drug administration and sampling is a major barrier for long-term pharmacokinetic study or repeated administration of inhaled pharmaceuticals.

Limitations of direct administration of drugs to anesthetized animals can be overcome by using passive administration chambers such as whole body, nose only and head only exposure chambers (Fig. 4). These chambers allow administration of drugs directly to conscious animals thus mimicking a more physiological condition (Dorato, 1990). While administration of drugs to conscious animals using various chambers offer a number of advantages, these methods also suffer from a number of limitations. Whole body exposure chambers, for example, do not require animals to be restrained to inhale drugs. But in this model, it is likely that drugs enter the animal body through other routes including oral and percutaneous routes. Further, since whole body exposure chambers are required to be operated at high flow rates, a larger dose is required compared to intratracheal instillation, nose only and head only exposures (Cheng et al., 2010). Furthermore, size of the animals, breathing patterns and lung capacity can lead to variations in pharmacokinetic and pharmacodynamic profiles. Whole body exposure chambers can be of different sizes, shapes (Wong, 2007) and made of different materials depending on the substance to be tested. For example, glass, polyester, Teflon®, PVC, polyethylene and polypropylene chambers are suitable if test materials contain chlorine and water vapor (Martin et al., 2003; Tian et al., 2008). High purity stainless steel, Teflon® and glass is preferred if test atmosphere contains ozone gas (Cheng et al., 2010). Since rodents are nose-breathers (Harkema et al., 2006), it has been suggested that nose only inhalation to conscious animals

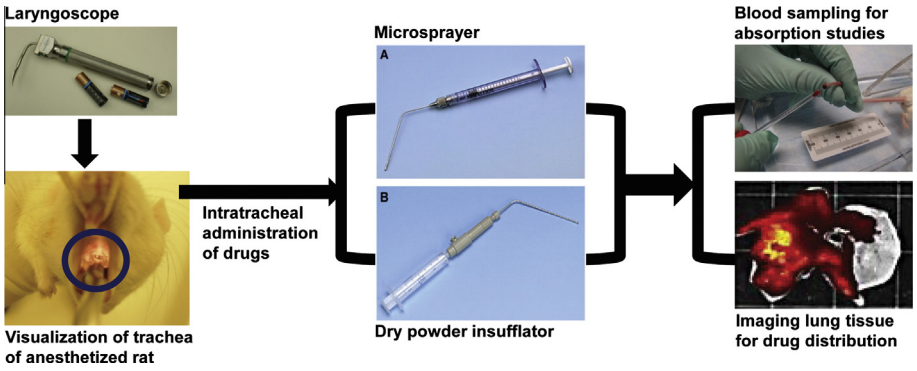

Fig. 3. In vivo model(s) for studying drug absorption profile following pulmonary administration. Upon visualization of the trachea using laryngoscope, drug is administered using a microsprayer or dry powder insufflators. Sample analysis can be performed by collecting blood or by fluorescence microscopy.

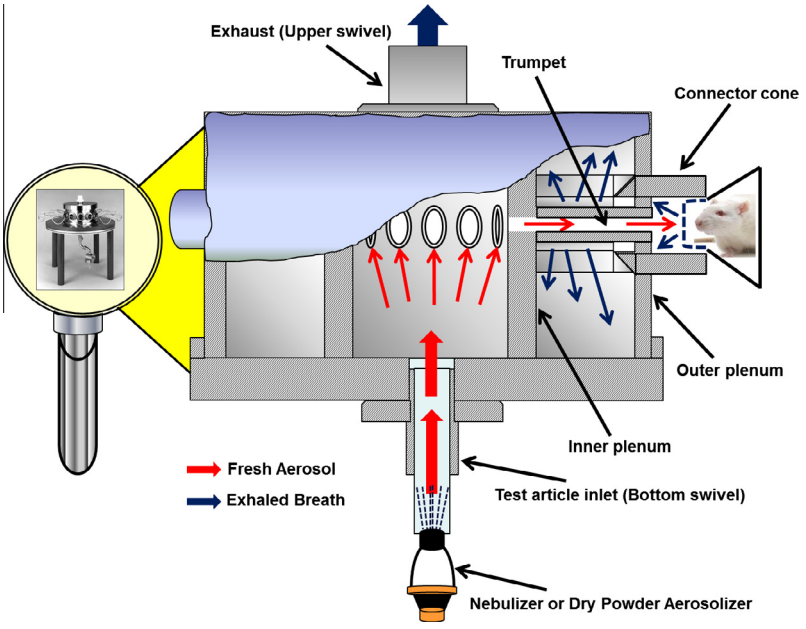

Fig. 4. Schematic diagram of exposure chambers used for nose-only exposure of drug/formulation to rodents.

perhaps would better mimic the physiological condition than forced intratracheal instillation under anesthesia. Compared to whole body exposure, nose only or head only exposure chambers allow entry of larger amount of drugs into the lungs because in these two chambers only nose or head is exposed to individual holding tubes. However, in nose only and whole body chambers animals are kept under stress in the holding tubes. Depending on the study design, all these three chambers are used in different experiments. Head only and nose only exposure system potentially eliminates other routes of exposure, required less test materials and are appropriate for repeated dosing studies. However, these two exposure chambers have some drawbacks: animals cannot get food and water during exposure, the system may generate heat, and animals may get suffocated in the chamber and require intensive labor (Wong, 2007). For nose only exposure, animals are placed in commercially available chambers that continuously generate aerosols (Lee et al., 1987). Although nose only exposure is more physiologically relevant, aerosols are deposited mainly in the nasal cavity that eventually is transported to GIT by means of mucocilliary clearance (Pauluhn, 2003). As a result, the dose reaching the lungs becomes much less compared to that administered by intratracheal instillation. Further, a significant wastage of drug and test materials has been reported to occur because of system inefficiency. CH Technologies (Westwood, NJ), Intox products (Edgewood, NM), TSE GmbH (Badhamburg, Germany) and ADG Developments (Herts, UK) commercialized various types of nose only chambers. Nose only exposure chambers are also suitable for various types drug delivery systems, such as liposomes (Mainelis et al., 2013), polymeric microparticles (Garcia-Contreras et al., 2006), and porous nanoparticles aggregate particles (Sung et al., 2009b). Depending on the experimental design, nose only exposure chambers are available for a small (Mainelis et al., 2013) and large number of animals (Pauluhn, 1994). Mainelis et al. has used direct flow nose only exposure chambers for a study which offers the advantage of eliminating further dilution of the exposure air by exhaled air of animals. In this study, a five port nose only exposure chamber manufactured by CH Technologies Inc (Westwood, NJ) was used for the treatment of lung cancer in mice. The design of this 5 port chamber is based on the 12 port nose only exposure chamber of the same manufacturer. This camber uses flow fast design that minimizes variation in the aerosol delivered to the port (Pauluhn, 1994; Pauluhn and Thiel, 2007). In a few studies, 12 port nose only exposure chambers have also been used (Port et al., 2004; Vincent et al., 1997). Cylindrical chamber with animal port located around the central chamber is a popular design for nose only exposure chamber, wherein test atmosphere enters from the top and reaches individual animal port via a tube (Fig. 4). Test atmosphere includes both animals housing conditions

(temperature, humidity, oxygen, noise level, light and presence of other animals) and exposure systems (Wong, 2007). Both single level and multilevel nose only exposure chambers are used for research purpose. In single level exposure chambers, test atmosphere enters on top of the animals head and leaves at the bottom of the animal restrainer tube (Stavert and Lehnert, 1990). Exhaled air also leaves with the exhausted air thus there is little chance of inhalation of this air by other animals. On the other hand in multi-level nose only exposure chamber, test environment enters the inner chamber and each animal inhales by individual port form the common chamber. Usually these types of chambers can accommodate large numbers of animals (Morris et al., 2005). To minimize rebreathing and cross contaminations, these chambers are designed in such a way that exhaled air enters the space between the inner and outer chambers thus leaves with the exhausted air (Cannon et al., 1983). Readers are directed to two excellent reviews by Cryan et al. and Sakagami for a detailed discussion with regard to the advantages and limitations of intratracheal instillation and nose-only exposure based models (Cryan et al., 2007; Enna and Schanker, 1972b; Sakagami, 2006).

3.2. Cell culture models for respiratory epithelium

Cell based *in vitro* models have been used extensively to study the uptake, transport and metabolism of drugs by the lungs (Hussain and Ahsan, 2006; Mitchell and Nagel, 2003b; Salomon et al., 2012) (Fig. 5) as they mimic microenvironment of the tissue. Over the past few decades, cell culture models have received increasing acceptability as an alternative to animal models because of a number of factors that include reduced cost compared to intact animals, ability to predict and simulate, and fast throughput. Further, 3R strategy (Replacement, Reduction and Refinement) recommends reducing animal usage, developing non-animal methods and improving scientific methods for animal welfare (Flecknell, 2002; Klein et al., 2011). Thus, *in vitro* models ranging from monocultures to double/triple cell co-cultures representing simple to more realistic three-dimensional (3D) models of lung tissue have been developed. Epithelium, covering the airway and alveolar regions, is the main barrier to drug absorption after pulmonary administration. So attempts have been made to develop cell lines that model both airway and alveolar epithelium. Two readily available human cell lines that have been used to model bronchial epithelial cells are 16HBE14o (Ahsan et al., 2003) and Calu-3 cells (Cavet et al., 1997). 16HBE14o cells were derived from bronchial epithelial cells of one year old heart lung transplant patient, and Calu-3 cells are developed from bronchial adenocarcinoma of the airway. Both cells form confluent monolayers with well differentiated epithelial morphology with tight junctions. These two cell lines are morphologically similar to that of native bronchial epithelium and produce a physical barrier to pulmonary drug absorption (Steimer et al., 2005). For transport studies, cells are grown in commercially available transwells with permeable filter support (Macdonald et al., 2013). A number of factors may influence the epithelial barrier properties or permeability of drugs under investigation. Growing cells in air–liquid interface and liquid only interface play a major role in transepithelial electrical resistance, permeability, morphology and mucus production and formation of cilia by the cells. Similarly, culture surface, matrix materials for surface coating, cell seeding density, composition of culture medium can affect both the monolayer properties such as confluence and Transepithelial Electrical Resistance (TEER) (Liedtke, 1988; Steimer et al., 2005; Wu et al., 1990). A detailed methodology about culture condition and morphology of the cells is beyond the scope of this review. For that matter, readers are referred to several excellent reviews covering *in vitro* cell models of respiratory drug delivery (Forbes and Ehrhardt, 2005; Steimer et al., 2005).

In addition to the epithelial cells, other cells such as dendritic cells and macrophages are also present in different generations of airways and these cells communicate with each other to interact with foreign particles. Co-culture of two or more cells have been used to predict interactions between particles and cells and to gain insight regarding lung environment. It has been shown that co-cultured cells respond differently to particles compared to mono-cultured cells. Co-cultures involve cultivation of two or more cell together and are generally characterized for their tight junctions and expression of surface proteins. A triple co-culture consisting of alveolar epithelial cells (A549), dendritic cells and macrophages has been grown and supplemented on membrane filter inserts. Uptake of polystyrene particles has been shown to be greatly influenced by the interplay between three different cells (Rothen-Rutishauser et al., 2005). This model have been shown to mimic air-blood barrier and provide information regarding cell–cell communications and mechanisms concerning cell-particle interaction and fate of inhaled particles. In a separate study, bronchiolar epithelial cells (16HBE14o) were cultured along with dendritic cells and macrophages to form triple co-culture model for studying interactions of cells with inhaled foreign particles. 16HBE14o cells were chosen because epithelial cells Type-I are more prominent than Type-II cells and hence offer more realistic representation of air-blood barrier (Lehmann et al., 2011). In a recent study, Jantzen et al., evaluated the influence of monocultures and co-cultures on the production of reactive oxygen species and oxidative stress-related damage to DNA upon exposure to particles. In this study, A549 cells were grown either alone or with macrophages. The results demonstrated that co-cultures exhibit less oxidative damage compared to monocultures suggesting the type of culture influence the response to xenobiotics (Jantzen et al., 2012). Since animals and humans are multicellular organisms, co-culture models comprising of different cells are more realistic and useful in studying air-blood barrier.

However, it is important to point out that, compared to the *in vivo* models, *in vitro* models of drug absorption offer reproducibility, simplicity, and better control of data acquisition along with reduced operation cost. However, there are still a lot of unknown factors which govern the process, including the mechanical processes of physiological breathing and the effect of breathing-

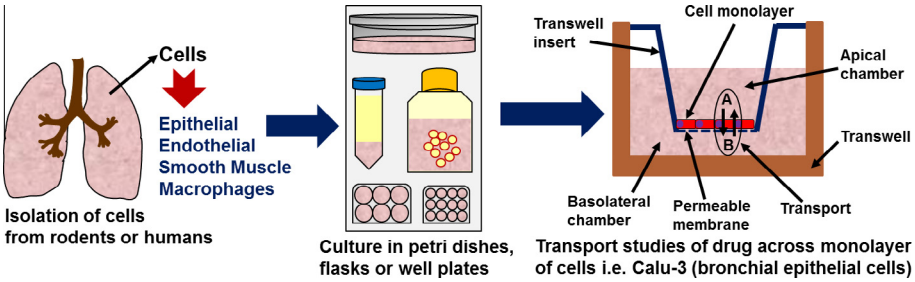

Fig. 5. *In vitro* cell culture model to study drug uptake, absorption and metabolism across the pulmonary epithelium.

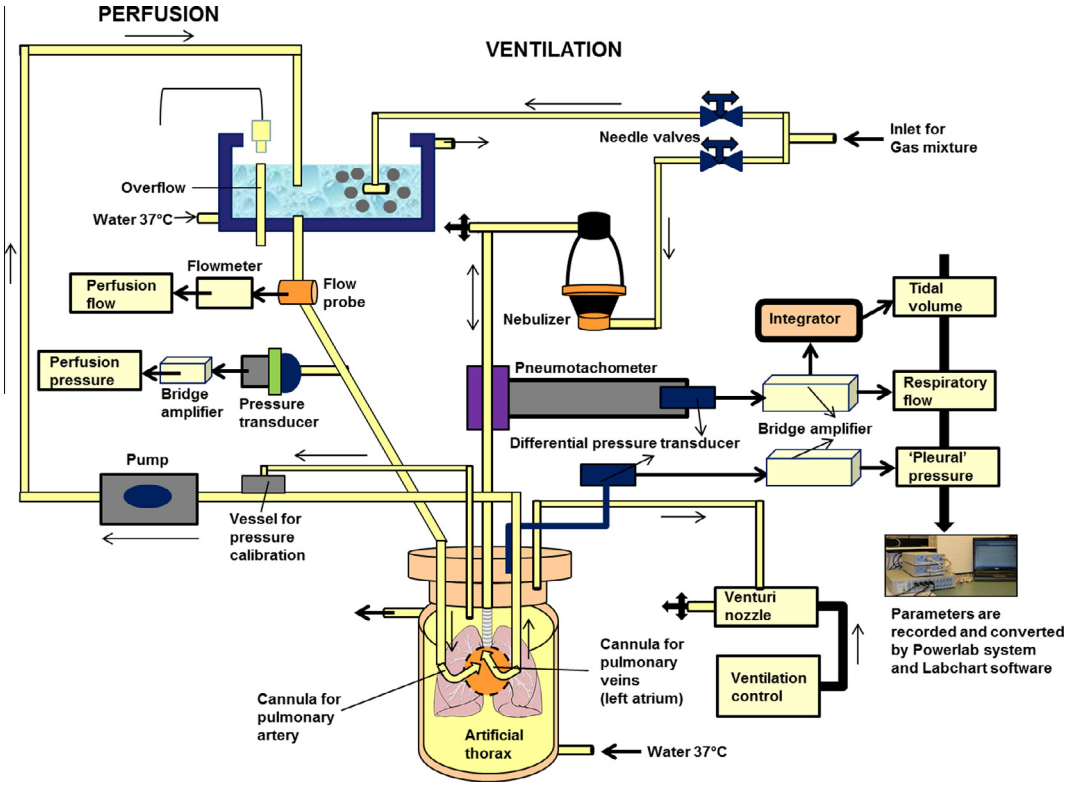

Fig. 6. Scheme of an Isolated Perfused Rat Lung (IPRL) System. Briefly, isolated lungs are perfused with physiological buffer via pulmonary artery and veins through a cannula while housed in an artificial thoracic chamber. The trachea is cannulated to maintain respiration of lungs under negative ventilation and administer drugs of interest.

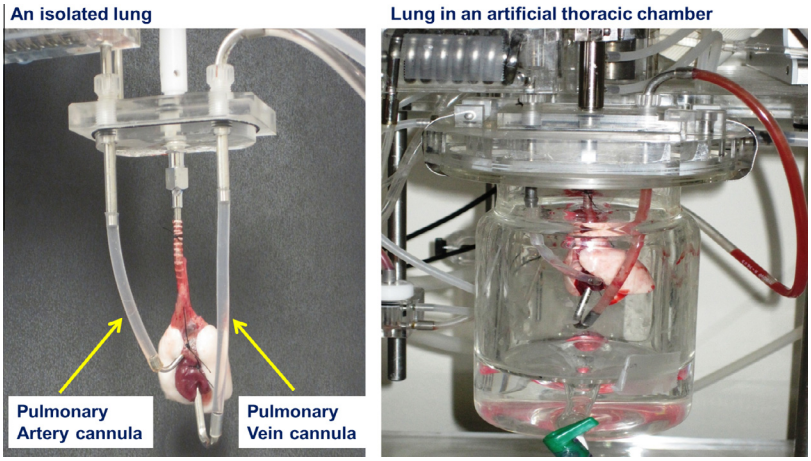

Fig. 7. Studying drug absorption profile using IPRL system. Cannulation of pulmonary artery and vein (left), artificial ventilation in a thoracic chamber.

associated stresses on lung cell responses to inhaled substances. Further, as the lung is made of 40 different types of cells, a co-culture set up that mimics the actual respiratory environment is yet to be established. Thus recently proposed tissue engineered lung models and polymeric chips mimicking the airway-capillary interface are likely to address many limitations of *in vitro* cell culture method. For detailed discussion on tissue engineered models, readers are directed to one of our recently published review articles (Patel et al., 2012c).

3.3. Ex vivo lung model

Ex vivo studies, such as the experimentation performed outside the organism in an artificial environment that mimics biological environment with minimum alterations, provide a thorough understanding of the physiological processes of the organ in question without complications associated with the use of whole body. *Ex vivo* models provide a means of evaluating various parameters in an intact organ, which would not be possible in intact subjects due to ethical and pathological concerns. One of the well established *ex-vivo* tissue models is isolated perfused tissue models, which allow studying the mechanisms of drug transport, disposition and efficacy in the isolated organ while maintaining the structural and functional integrity of the organ. Isolated perfused tissue models provide a more realistic correlation with the *in vivo* studies compared to the single cell monolayer models. Isolated perfused lung (IPL) models have been developed for rodents such as rats (Byron et al., 1986; Niven et al., 1990) and rabbits (Bleyl et al.,

2010; Piacentini et al., 2008), while attempts have also been made to establish human lung perfusion models (Briot et al., 2009; Linder et al., 1996).

The isolated perfused rat lung model (IPRL) has been used for studying absorption and deposition of inhaled pharmaceutical formulations. This method involves surgical removal of the lung and placing it in an artificial thoracic chamber supplied with atmospheric air and perfusion media. The surgical procedure for isolation of the lungs is fairly complex and requires extra caution to prevent any tissue damage. Briefly, anesthetized animal is placed on a surgical platform and a long incision is made from abdomen to the neck. The trachea is then identified, cleared for adhering tissues and cannulated using tracheal ventilation cannula to provide respiration to the lungs and to administer drugs of interest. Positive pressure ventilation is provided to prevent collapse of the lungs during its surgical removal from the body. Subsequently, the thoracic cavity is exposed by making a vertical incision along the sternum. Acannula (inlet) is then inserted into the pulmonary trunk through a small incision at right ventricle. Next, a longitudinal incision is made at left ventricle close to the heart apex parallel to the interventricular septum and a second cannula (outlet) is inserted into the left atrium through mitral valve to collect perfusion medium. Then, the lung is carefully detached from thorax and placed in the artificial thoracic chamber of the IPRL setup. Finally, the positive-pressure is changed to negative-pressure ventilation to mimic physiological processes (Chiang et al., 2012). Figs. 6 and 7 show schematic diagram of IPRL instrument and lungs housed in the artificial thoracic chamber, respectively. Following surgery, drug or formulations can be administered either in perfusate or directly into the lungs using suitable delivery devices in order to evaluate various pharmacokinetic parameters without the influence of whole body. In IPRL, a variety of perfusate media including physiological salt solution-ficoll and Krebs-Henseleit solution containing albumin with or without hematocrit have been investigated. Generally, before performing surgery, perfusion medium is filtered and then recirculated in the IPRL instrument for the adjusting to physiological pH (7.4) and temperature (37 °C) and is maintained throughout the experiment. Various drug delivery systems such as metered dose propellant based intratracheal dosing cartridge, nebulizers, inhalable dry powder exposures and the Dust Gun aerosol technology have been investigated for drug administration into the isolated lungs (Byron and Niven, 1988; Ewing et al., 2008; Selg et al., 2010).

Pharmacokinetic behavior and deposition of various drug entities including small molecules and proteins have been studied using IPL models. Ewing and coworkers have reported detailed pharmacokinetics of three anti-asthmatic drugs using IPRL after pulmonary administration of dry powders (Ewing et al., 2008). Recent studies investigating deposition, absorption and metabolism of inhaled insulin using IPL model have concluded that these process may be lung-region dependent and could be significantly influenced by self-association characteristics of the insulin molecules (Pang et al., 2005, 2007). Further, a study showed an isolated lung exhibiting clinical responses such as vasoconstriction following exposure to budesonide and suggested that this model can be employed for rapid screening of inhaled corticosteroids (Ewing et al., 2010). In addition, potential applications of an isolated perfused lung have been investigated in the area of toxicology and absorption of inhaled pollutants such as diesel soot and benzo(a)-pyrene (Ewing et al., 2006; Gerde et al., 2004).

Following drug administration, continuous sampling is possible from the perfusate outlet from the lungs. This method allows regulation of lung volume, ventilation rates, and respiratory patterns. In addition, recently generated data from IPRL sampling has also been utilized for *in vivo* kinetics of pulmonary drug disposition process (Sakagami, 2006; Selg et al., 2012). In addition to the small rodents, human IPL models have also been developed to study the molecular events taking place during the drug therapy, and also to keep the lungs alive before transplantation (Briot et al., 2009). However, there are several limitations associated with IPL models. One of the main concerns is the viability of tissues over a period of time. Keeping the lungs viable for more than 2–3 h at 37 °C has been quite challenging, chiefly due to edema formation. Some groups have reported up to 4 h of viability with careful handling and perfusion techniques (Orton et al., 1973). It has been noted that IPL model usually predicts the drug deposition in a lung perfused only by pulmonary circulation, as the bronchial circulation is usually severed during the surgery (Mehendale et al., 1981). Thus, this model should not be used to predict the upper airways drug deposition, rather other *ex vivo* methods including isolated tracheal tissues should be used (Rasmussen and Bhalla, 1989).

The above discussion suggest that while there are a number *in vitro*, ex vivo and *in vivo* models no single model is fully capable of predicting all the parameters for deposition and absorption of inhaled pharmaceuticals, which makes it necessary to utilize one or more models that complement each other.

4. Conclusion

Various experimental tools continue to emerge since the pulmonary delivery systems have become popular as an effective strategy to administer therapeutic agents. Various *in vitro, in vivo* and *ex vivo* models discussed in this review are instrumental in determining particle deposition patterns and absorption profiles of inhaled therapeutics and hence are being extensively used. However, there is no such model that can exactly mimic the complicated human lung microenvironment. The emergence of *in silico* and bioengineered lung models aim to come up with a closely resembling system to the human lungs, however, such systems are still in their infancy. It will be interesting to follow how the current research, which is extensively moving towards a better model to study post-administration behavior of inhaled drugs, pave the way for a system where the fate of therapeutic agents administered to humans via the pulmonary route could be evaluated with desired precision and reproducibility.

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