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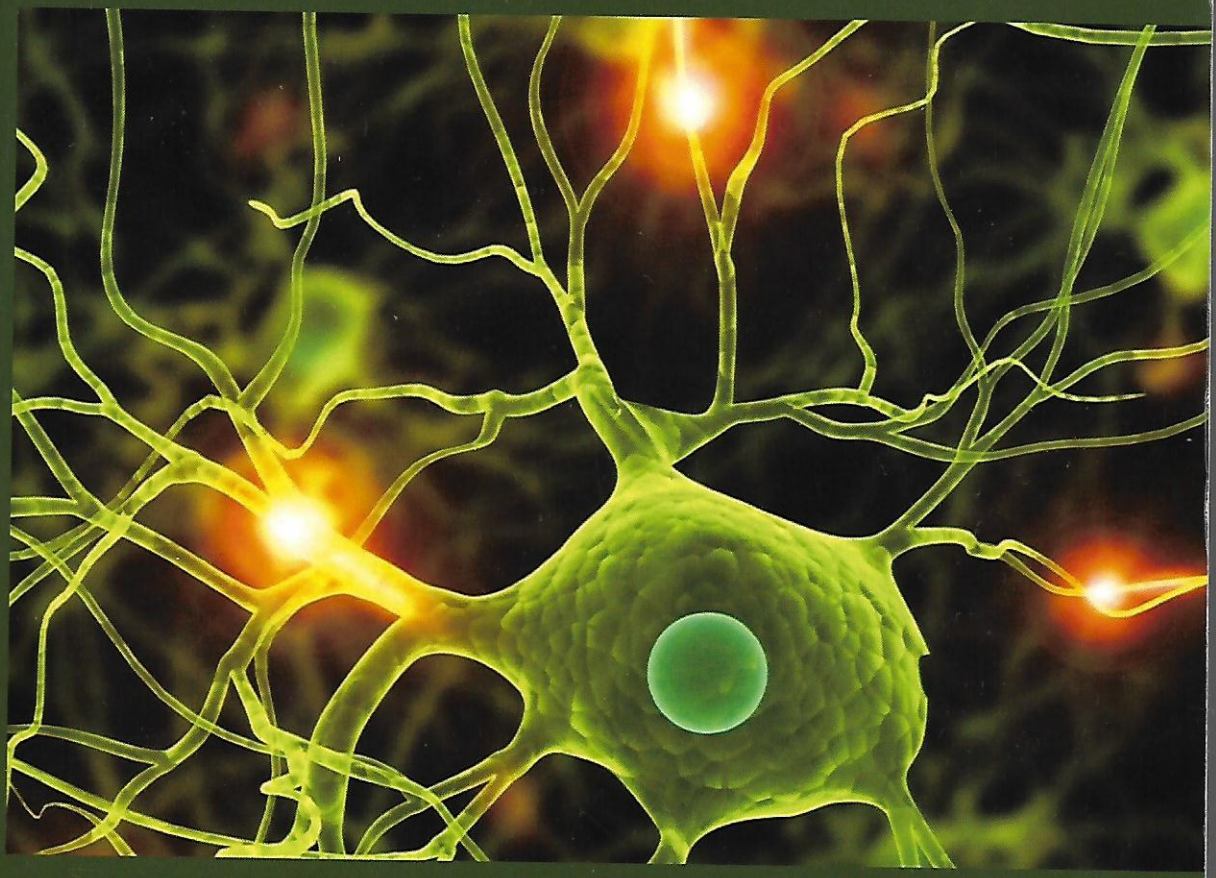

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Dr. Shaon Ray Chaudhuri

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Editor's Biography :

Dr. Shaon Ray Chaudhuri did her PhD (2001) from Department of Biophysics, Molecular Biology and Genetics, Calcutta University after finishing the bachelors (1994) and Masters (1996) in Physiology from Calcutta University. She worked as DBT PDF in Life Sciences at Jadavpur University (2001-2003). She received the young scientist award under the DST fast track scheme in 2003 and continued to work at Jadavpur University till she joined West Bengal University of Technology on November 2004. She did post-doctoral training at Technical University of Munich, Germany as well as Humboldt University, Berlin.

Between November 2004 and August 2015 she setup the Microbial Technology Group and took it to the height of obtaining Centre of Excellence in Environmental Technology and Management from Ministry of Human Resource and Development, Government of India under the FAST scheme in 2014. She has filed 12 patents (including international patents) and transferred three technologies to industry. She has 46 papers while 10 book chapters. Five scholars have obtained their PhD, two others are awaiting their final defence post submission; two others are expected to submit their thesis in 2016 while four others are currently working in the group under her guidance. She has obtained extramural grants to the tune of Rs 5.828 Crores from different granting agency till March 2016. She moved to Tripura University as Associate Professor in the Department of Microbiology on 1st September 2015 and is instrumental in obtaining the first faculty grants for the department, signing of MoU with industry and institutes as well as syllabus revision for the existing MSc course with support from faculty members from the different departments of the University.

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CONTENTS

CHAPTER - 2

A GLIMPSE ON TUBERCULOSIS AND SCOPE OF INHALABLE ANTI TUBERCULAR THERAPY

Vishnu Vardhan Reddy Beeram[1] and Venkata Nadh Ratnakaram[2]*

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Abstract

Tuberculosis (TB), is an airborne and notorious disease caused by Mycobacterium tuberculosis. Despite the technological advances, the tubercle bacillus continues to threaten humans. Most patients are noncompliant towards current modes of treatment due to resistance of bacilli for single or multiple drugs. The usual treatment duration ranges from 6-9 months for drug susceptible TB; 18-24 months for multidrug resistant TB. However the long term treatment posses adverse events. To avoid the adverse events and improve the efficacy, alternative modes of drug delivery systems are desired. Among them, the nanotechnology overcomes the limitations of conventional therapy and improves the efficacy. The nanocarriers can be considered for pulmonary application by virtue of their potential advantages. However, nanoparticles meant for lung delivery poses certain challenges such as difficulty in characterization and the extent of utility towards practical applications. It must be noted that the development of nanocarriers for pulmonary application requires an in depth knowledge of the lung, both in its healthy and disease state. Recent developments include in vitro models based on cell line cultures. The present paper describes the development and characterization aspects for inhalable drug delivery systems for the targeted and slow delivery of antitubercular drugs.

Keywords: Tuberculosis, Mycobacterium tuberculosis, Drug delivery systems, Inhalable drug delivery system, Targeted delivery, Anti-TB drugs

INTRODUCTION

Hippocrates (460-377 BC) identified the process, phthisis (or consumption). It is the process where the substance is completely destroyed and was the most widespread and lethal disease of its time (Soled, 1991). The tubercular decay was found in 3000 B.C in Egyptian mummies (Zink et al., 2003) and further evidence that tuberculosis (TB) has accompanied mankind for a very long time. During the 18th and 19th centuries the disease ravaged Europe and North America earning the nickname "Captain among these men of death". The Europeans and Americans during 18th and 19th centuries began to understand the pathophysiology of the disease (Daniel, 2006). Robert Koch identified *Mycobacterium tuberculosis* as the causative agent for TB in 1882 (Koch and Pinner, 1932). About 40 years later, Albert Calmette and Camille Guerin had developed a vaccine (BCG) by serial passage of the closely related species *Mycobacterium bovis* (Calmette, 1928). About four new frontline anti TB-drugs (ATDs) were introduced during 1952-1961, the drugs when used in combination has shown success rate of up to 87% (Global Tuberculosis Report, 2012). Eventually the research on TB has grown and at present, 11 new vaccines and several TB-drugs are available. Some drugs are in clinical trials and under regulatory review. Despite the continual effort to develop tools against the disease, about 9 million people are reported to have TB. About 1.5 million people were died due to TB, and among them 360,000 people were HIV positive (Global Tuberculosis Report, 2012). Furthermore, it is estimated that about one third of the world's population is latently infected with TB (Enarson and Ait-Khaled, 1996). The review describes the fundamentals of infection and its development, inhalable drug delivery systems based on nanoparticles and their characterization.

INITIAL INFECTION

The TB is an airborne disease, initially spread from the infected host by coughing or sneezing in the form of droplets containing the bacilli upon inhalation (Turner and Bothamley, 2015; Aliabadi et al., 2011; Tuberculosis fact sheet, 2012). The bacilli reach the site of gas exchange, the alveoli, and a void being trapped in the mucosal layers of the upper respiratory tract. The professional phagocytes, alveolar macrophages engulf the bacilli (Dannenberg, 1993) (Fig-1) and prevent the infection in general. However the bacilli dominate and spread the disease in susceptible individual. There exists an involvement of diverse receptors for the uptake of the bacteria that include Toll-like receptors (TLR), cluster of differentiation 14 (CD14), complement, mannose, SPA and scavenger receptors.

In addition, next target of bacilli include Fc receptors after onset of adaptive immunity (Ernst, 1998; Dunne et al., 1994). It was believed that the macrophages infiltrate the engulfed bacilli into sub lying epithelial cells causing a local inflammation (Ulrichs and Kaufmann, 2006). The inflammation eventually show affinity towards the mononuclear cells from the nearest blood vessels, thus providing a new environment of host cells for enhancing the bacterial population (Davis and Ramakrishnan, 2009) thus the disease is further expanded. The early lesions form the mucosa for eventually develop into tubercle or granuloma, the hallmark structure of TB (Adams, 1976). Alternatively, the bacilli transported to nearest lymph via dendritic cells (DC) for activation of the adaptive immune system (Wolf et al., 2007). It was believed that the lymphohematogenous route was the path for the establishment of secondary TB lesions (Cooper, 2009). Another form, miliary tuberculosis is lethal as the mortality rate is high and it mostly affects the young children and patients with immunodeficiency (Harries, 2004), is 30% even when treated (Hussain et al., 2004). However the prime location of the infection in most of patients (80-85%) is the respiratory tissue (Lawn and Zumla, 2011). The life cycle of Mycobacterium tuberculosis is shown in Figure-1.

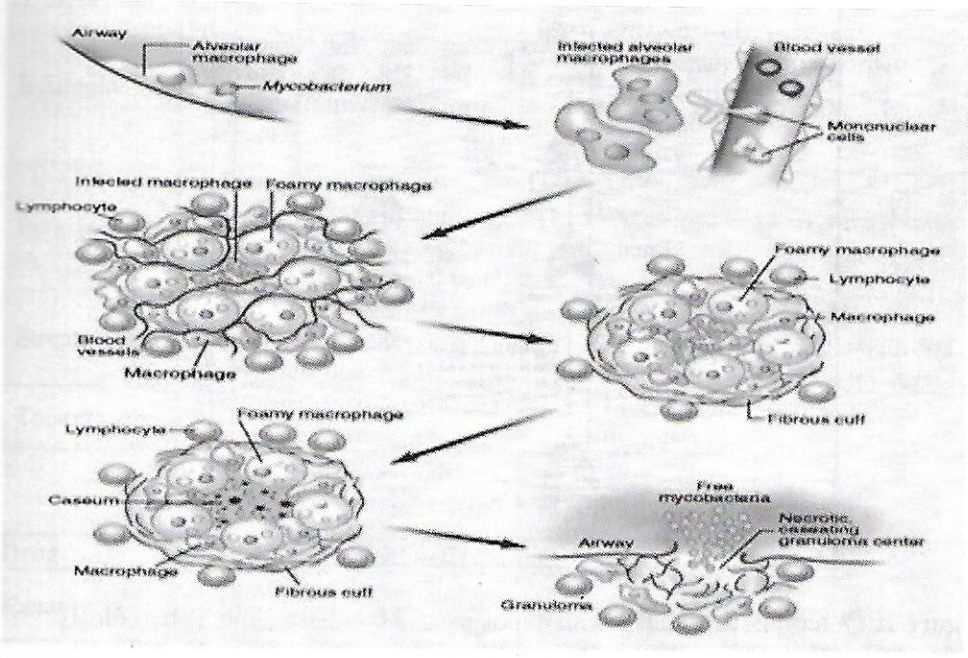

Figure 1- The life cycle of Mycobacterium tuberculosis showing the bacteria uptake followed by granuloma formation, accumulation of necrotic cell and granuloma rupture (David et al., 2009).

The occurrence of initial infection is as follows

Primary progressive TB: Forty percent of infections are associated with the primary active TB, in which the immune system of the host is unable to destabilize the bacilli (Parrish et al., 1998) (Fig-2). The initial infection gradually expands and causes infections eventually results in complete destruction of the infected organ (Harbitz, 1922). When the disease is left untreated, the mortality rate of progressive TB is about 66% (Global Tuberculosis Report 2012).

Latent TB: The latent TB is a state of latency, a non transmissible form in which clinical signs of disease are absent (Russell et al., 2010) and it persists in about 60% of cases. It is estimated that one third of the world's population carry this latent version of TB (Enarson and Ait-Khaled, 1996). The majority of patients live their lives without ever knowing that they carry the disease. However some (2 to 23% lifetime risk) of them will experience the reactivation of the infection to secondary active TB. The number of cases (5-10% annual risk) is relatively high in the people with compromised immunity due to HIV/AIDS or the use of immunosuppressant (Gedde-Dahl, 1952).

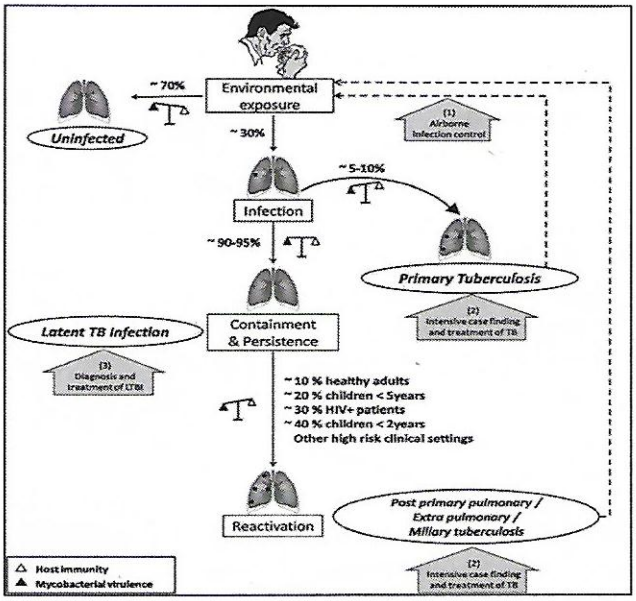

Figure 2: Outcomes associated with exposure to Mycobacterium Tuberculosis (Davies, 2001)

NOVEL AND MODIFIED RELEASE DELIVERY SYSTEMS

TB has been a leading killer disease globally and a multiple of first-line ATDs must be administered regularly for at least 6 months to relieve the disease. The use of antimycobacterials since decades is in practice. However the control with

antimycobacterial chemotherapy is a tricky and arduous task mainly because (i) the therapy involves long term administration, (ii) poor permeability and thus less availability of antimycobacterial drugs at target site, (iii) all antimycobacterial drugs are highly toxic, and (iv) significant patient noncompliance to prescribed medicines is observed especially due to the length of the therapy and also due to the therapy associated severe side effects. Numerous side effects are reported for antitubercular drugs (Table 1 and 2). The dangerous side effects include hepatotoxicity, neurotoxicity and ocular toxicity. Most of the side effects are attributable to the inadequate modes of drug administration and thus present a great challenge for drug delivery technology and for the scientific community including formulation scientists (du Toit et al., 2006). To overcome, the limitations of conventional modes of drug delivery alternative modes of drug delivery systems and their administration would be desired.

Table 1: Side effects of first line Antituberculosis drugs

Drug	Major adverse effects	Rare adverse effects
Isoniazid	Peripheral neuropathy, skin rash, hepatitis, sleepiness and lethargy	Convulsions, psychosis, arthralgia, anemia
Rifampin	Abdominal pain, nausea, vomiting, hepatitis, generalized cutaneous reactions, thrombocytopenic purpura	Osteomalacia, pseudo-membranous colitis, pseudo-adrenal crisis, severe renal stoppage, hemolytic blood paucity
Pyrazinamide	Arthralgia, hepatitis, gastrointestinal problems,eg, stomach upset, nausea, poor appetite and abdominal pain	Cutaneous reaction, sideroblastic anemia
Streptomycin	Vestibular and auditory nerve damage renal breakage, cutaneous allergic reaction	Pain, rash at injection site, numbness around the mouth and tingling soon after the injection
Thiacetazone	Skin rash that sometimes has mucosal involvement	Liver failure, dermatological disorders

Table 2: Side effects of second line Antituberculosis drugs

Drug	Major adverse effects	Rare adverse effects
Kanamycin	Vestibular (vertigo) and auditory nerve damage	Cutaneous hypersensitivity
Amikacin	Vestibular damage (vertigo) and auditory nerve damage	Clinical renal failure
Capreomycin	Nephrotoxicity	

Ethionamide (prothionamide)	Gastrointestinal anorexia, nausea, diarrhea, abdominal pain, hepatotoxicity	Convulsions, mental symptoms, impotence, gynecomastia
Fluoroquinolones	Gastrointestinal anorexia, nausea, vomiting	Anxiety, dizziness, headache, convulsions, rupture of the Achilles tendon
Cycloserine	Dizziness, headache, depression, psychosis, convulsions	Suicide, generalized hypersensitivity, hepatitis
Para amino salicylic acid	Gastrointestinal anorexia, nausea, vomiting, hypersensitivity reactions (fever, rash, pruritus)	Hypothyroidism, hematological reactions

Nanotechnology is the promising technology and has been a boon to current pharmacology and biopharmaceutical enhancement of drug performance. It is possible to design drug delivery systems capable of targeting phagocytic cells that are infected by intracellular pathogens, such as mycobacteria. The delivery systems based on nanotechnology offer wide opportunities for improving therapy efficacy for a wide range of diseases including TB (Fig-1). Therefore alternative modes of delivery is required to develop the devices for the enhancement of bioavailability and site specific targeting of first line ATD therapeutics to avoid development of drug resistance (Schatz et al., 2005). The bioavailability can be enhanced by bypassing the barriers that hinder the bioavailability. Diverse approaches for the development of nanoparticles have been reported for the exclusive delivery of chemotherapeutic agents to target sites. This helps in improving the therapeutic index of the drug by stringently localizing its pharmacological action to the target site or organ. Thus it may be said that the nanoparticulate drug delivery systems help in improving the tolerance for toxic chemotherapeutics a part from the bioavailability enhancement (Shegokar et al., 2011)

INHALABLE DRUG DELIVERY

Lungs are an attractive target for the pulmonary administration of therapeutic moieties in the form of various drug delivery systems (Azarmi et al., 2008; Jaafar-Maalej et al., 2012). Additionally, the pulmonary route offers advantages over per oral route that include high surface area with rapid absorption due to high vascularization and avoidance of first pass effect (Sung et al., 2007). This selectivity allows targeted drug delivery and, hence, reduces the side effects (Beck-Broichsitter et al., 2009: Beck-Broichsitter et al., 2012). Colloidal drug delivery systems have extensively been investigated as drug carrier systems for the application of different drugs via different routes of administration. Solid lipid

nanoparticles (SLN) are one of the most interesting colloidal systems that have studied for more than a decade ((Mueller, 2000; Mueller, 2008)]. SLN are nanoscale suspensions composed of phospholipids and triglycerides with physiological tolerability (Morimoto et al., 2013; Nassimi et al., 2009). Apart from SLN, biodegradable polymeric nanoparticles are also attaining importance due to the sustained release properties. The SLNs are ideal drug carriers for drugs with poor aqueous solubility and high lipophilicity. The increased solubility in lipophilic matrix adds a positive effect in terms of improved pharmacokinetics and efficacy. Nanoparticle based drug carriers open up new initiatives such as modification of physical properties. The parameters include enhancing drug solubility; drug loading capacity and surface modification to modulate the drug release behavior (Paranjpe et al., 2013; Menon et al., 2014). Certain nanosystems also possess toxic effects apart from their therapeutic effects. The toxicological testing of naoparticulate systems is necessary to assess the safety of nanoparticles utilizing *in vitro*, *ex vivo* and *in vivo* cell culture models. The testing is essential to assess the risk potential of nanoparticulate systems. The choice of the inhalation device in a specific patient population also plays a vital role in nanoparticle-mediated drug delivery systems. Various parameters and their relationship shall be considered in the development of nanoparticles as illustrated in Figure 3.

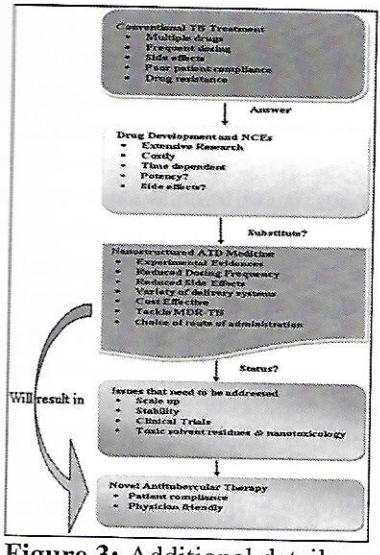

Figure 3: Additional details to support the utility of nanoparticles in TB

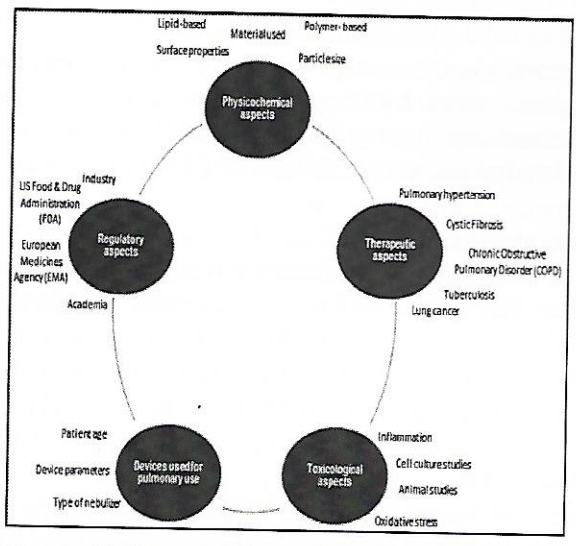

Figure 3: Complex interplay of parameters in the research and development of pulmonary drug delivery systems.

Physicochemical Characterization

Particle size and charge measurements

Particle size and charge (zeta potential) measurements are important for the characterization of nanoparticles in order to ensure the optimal particle size distribution and polydispersity index (PDI). Photon correlation spectroscopy (PCS) and laser diffraction (LD) are widely used method for the estimation of particle size. PCS measures the particles ranging from few nanometers to maximum of 3 μm. A beam of light is passed on the nanoparticles dispersion (Lim et al., 2013). The scattered light from the nanoparticles is measured, amplified and correlated to size. PCS can also measure the PDI, which determines the uniformity of the particles. A higher value of PDI (>0.2) normally indicates multiple sizes of particles in the given formulation, thus smaller PDI is desirable for uniform distribution of particles. The LD technique measures particles with a bigger size and is based on the measurement of the diffraction angle depending on the particle size. LD measures particles from the nanometer to a few millimeter size ranges (Kelly, 2004; Tena and Clara, 2012). It is recommended to use both techniques simultaneously. The zeta potential measurements are essential for understanding the stability behavior. The high values of zeta potential contribute for repulsion and make the particles to less aggregate. In addition, the uniform sized particles show good stability.

Differential Scanning Calorimetry (DSC)

DSC is used to detect the polymorphic changes if any in the lipid matrix. The structural changes in the lipid matrix provide information about the instability of delivery system. Melting and recrystallization curves are appropriate parameters to determine polymorphic changes of the lipids. Bunjes *et al* (2010; 2011) reported the detailed analysis of the use of DSC in lipid-based systems.

X-ray diffraction

X-ray diffraction studies analyze the crystal structure in terms of spaces in the lipid lattice. The presence of drug in lipid matrix influences the lipid/polymer structure and thus the lattice spaces. The method can be used along with DSC, analyzes the patterns of the spaces over time. Hence, both techniques are recommended to use for the assessment of crsytalinitiy in lipid-based formulations (Bunjes, 2007).

Other techniques

More advanced methods, nuclear magnetic resonance (NMR), Raman spectroscopy and infrared spectroscopy are used for physicochemical evaluation. The techniques characterize specifically mixed systems associated with co-existence of micelles, liposomes and liquid crystals.

Techniques for particle morphology

The morphology of the nanoparticles can be examined by using transmission electron microscopy utilizing different techniques suitable for specific particles. Freeze-fracture, negative staining and cryogenic-transmission methods can be adapted according to the type of delivery system. The morphological determination is required to understand the structure, shape and alignment of the particles (Bunjes, 2011).

Cell and animal based studies

Several studies are reported (Ehrhardt et al., 2004) for the toxicity assessment utilizing various models that include cell lines, tissue and animal models. Toxicity studies are required to determine the lethal dose and the therapeutic window of the drug-loaded nanocarriers. Studies to demonstrate the therapeutic efficacy have been reported using various models. The *in vitro* models utilizing epithelial cells of respiratory tract for the characterization of nanoparticles were documented (Foster et al., 2000). The models involving an air liquid interface (ALI) have been extensively used to study the effects of various formulations intended for pulmonary application (Foster et al., 2000).

In vitro testing using cell culture models

In vitro cell culture models serves as primary testing models before initiating the *ex vivo* and *in vivo* tests. The advantages of the models include uniformity of thickness in cell layer; simple to handle and wide range of availability. The cell culture models facilitate the experimenter to plan multiple experiments and avoid the use of living animals. Diverse sources of pulmonary tissue are used to derive the cell models. Murine and animal pulmonary tissue is used for the establishment of models over decades (Forbes et al., 2003; Sakagami, 2006). The main aspect is to develop a standard cell line that can predict the transporter mechanisms across the pulmonary epithelium in a similar fashion as the Caco-2 cell line. A human bronchial epithelium cell line (16HBE14o) has been used for a long time to investigate the drug transport mechanism in across pulmonary membrane. Forbes *et al* (2003) established the cell line for studying transport of mannitol and analyzing the TEER values, observed that the higher permeability of hydrophilic molecules compared to the typical alveolar epithelium cell models. Furthermore, a sigmoidal relationship was derived between permeability and lipophilicity.

Another cell model was developed by Foster et al. (2001) based on sub-bronchila gland cell line, Calu-3. The parameters of experiment are investigated and concluded that the TEER values are necessary in terms of a tight monolayer for drug transport and efflux. Since many years, the model (Calu-3) was in use for drug permeation studies for low molecular compounds to be delivered via pulmonary route. The cell monolayer expresses a protein called as 'the cystic fibrosis transmembrane conductance regulator' protein. The cell membrane can

also be used for studies related to cystic fibrosis. The Calu-3 cell cultures produced using transwell plates with two chambered compartments. One chamber serves as a basolateral compartment to hold the culture medium and second is apical chamber. Other cell lines obtained from human pulmonary epithelium (A549 cell line); rat tracheobronchial and primary cells; rabbit alveolar-type cells and many (Sakagami, 2006) are used for *in vitro* testing

Ex vivo lung tissue models

Ex vivo lung tissue models have been used extensively along with the *in vitro* and *in vivo* characterization models (Paranjpe and Muller-Goymann, 2014). The models can be used to investigate the mechanisms of drug permeation across pulmonary tissue. Different models were established, such as isolated perfused lung (IPL) and precision cut lung slices (PCLS). IPL are obtained from rats. Mice are rarely preferred due to the small size and the complex isolation procedure. In addition, rabbits and guinea pigs can also be used. The IPL model consists of an isolated lung from an animal, immersed in assembly that resembles physiological conditions. The assembly contains physiological buffer, Krebs–Ringer or Krebs–Henseleit solution. The whole set up is maintained at a 37° C. The perfusate flow is maintained from 12 to 15 mL/min. The solution is equilibrated with oxygen and carbon dioxide to facilitate the function of the lung. By virtue of IPL structure, show resemblance to in vivo system in terms of physiological properties of lung tissue. The model also eliminates the pre-systemic metabolism. However, IPL possess limitations such as requirement of skill for the isolation of lungs in intact from the animal; skills in mounting; complexity of maintaining the experimental physiological condition. The IPL model with a detailed experimental set up and challenges is described by Sakagami (2006). The model was utilized for the investigation of nebulized 5(6)-carboxyfluorescein (CF)-loaded polymeric nanoparticles (Beck-Broichsitter *et al.* 2009), in order to assess the absorption behavior of CF nanoparticles from the perfusate solution. A rabbit IPL model was used for the comparison of CF in the form of nanoparticles *vs.* solution (Beck-Broichsitter *et al.* 2009). They also analyzed the physicochemical aspects of CF-loaded nanoparticles, such as the particle size and nebulization performance. It was found that the nebulization of CF nanoparticles had no influence on the particle size and the polydispersity index. Another most convenient *ex vivo* model is precision cut lung slices (PCLS) from murine models. The method utilizes the lungs filled with agarose solution, maintained at cold conditions for the gelation of agarose and facilitates the slicing of the lungs to desired thickness. To ensure, the viability of the lungs, the whole process is performed at low temperature in culture medium. After slicing, the agarose solution is washed using culture medium to remove traces of agarose. Desired sizes and locations of the slices can be done in consideration to alveoli and pulmonary vessels. Such slices can be used for the investigation of drugs that show contraction and relaxation of the pulmonary vessels. Advanced techniques such as microscopy coupled with

software may be used to examine the rate and extent of contractions and relaxations. The slices are the representative sections of lung tissue; retain the viable receptors and the physiological properties for three days. Sildenafil loaded SLN are investigated for toxicity test in comparison to plain SLN using PCLS (Neuhaus, 2013; Kaneko and Coppen, 2012).

In vivo models

The animals used for drug absorption and disposition studies include small rodents, mice, rats and guinea pigs. The use of mice as the animal model poses challenges to the experimenter for collection of blood samples due to their lung dosimetry. The larger animals, pigs, rabbits, sheep and monkeys can also be considered for inhalation pharmacokinetics however they are associated with high cost. The method involves incision of trachea using endotracheal tube; intratracheal instillation of about 10-200 µL of an aqueous suspension or solution using a micro syringe (Sakagami, 2006). The method requires skills in tracheotomy surgery. Alternatively, animal can be directly exposed to the aerosol in a chamber where the nose of the animal is fixed and the animal inhales the released aerosol. After inhalation for a defined period, the bronchoalveolar lavage fluid (BAL) is analyzed for the active drug. Some researchers investigated the mice after exposing to SLN aerosol (16 days treatment) for BAL fluid and cytokines (Nassimi et al., 2010; Morimoto et al., 2013). Furthermore, multiple blood samples from the animal are essential in order to determine the pharmacokinetics. In comparison, the nose is the only model easier for the animal and enables the investigator to have flexibility. The experiment with the estimation of BAL fluid is relatively faster than a pharmacokinetic study (Yapa et al., 2013). In addition, various experimental parameters should be taken in to consideration that includes breathing rate, dimensions of device, flow rate, variability and transport mechanisms. Many *in vivo* models have been established with respect to different lung disease conditions (Nemmar et al., 2013). Alternative models for in vitro investigation include monocrotaline sodium-induced pulmonary hypertension; models meant for investigation of anti-tuberculosis formulations; lipopolysaccharide or acrolyn induced airway inflammation models; and murine models for the production of mucus (Wang et al., 2009; Marsboom and Janssens, 2004). *In vivo* animal experiments are particularly important in preclinical studies and help to determine the safe dosage range of a formulation (Paranjpe and Muller-Goymann, 2014) for the subsequent Phase 1 of the clinical trial on humans.

CONCLUSIONS

The drug delivery via pulmonary route is rapidly emerging due to numerous merits over conventional modes of drug delivery. Abundant surface area of the lungs and the avoidance of the presystemic metabolism make the drug transport relatively faster in the lungs. Further the colloidal systems composed drugs with poorly

aqueous solubility, by virtue of their small size possess numerous advantages in drug targeting. The lipid-based drug delivery carriers such as SLN and liposomes show added advantages in terms of physiological compatibility hence many immunological reactions cannot be anticipated.

An extended release effects may be shown by biodegradable nanoparticles, although the drug release mechanism are in establishment stage. The stability aspects of nanoparticles are substantially established and the safety studies are in progress. Certain liposomal formulations in freeze dried form are developed and the safety and efficacy studies are under way. A successful formulations based on nanoparticles is anticipated for commercialization for inhalation delivery. The parameters of inhalators should also be focused parallelly apart from formulations. The investigations based on cell culture models pay an equal importance to prove the safety of the drug. The establishment of in vitro-in vivo or ex vivo- in vivo correlations poses challenges to the scientist. The models are desired to minimize certain clinical studies. The gray area of research is the assessment of pharmacokinetic behavior of drug via lung delivery, the investigations are further explored. The studies involving use of markers such as radioisotope and/or fluorescent labeling may allow the scientist to trace the drug transport into the cell. Finally, it can be concluded that the pulmonary research has wide scope for the success of nanocarriers based formulations.

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