1. Introduction

The rapidly evolving microbial resistance against the existed antibiotics has created an alarming threat to the public health globally. According to the World Health Organization report, nearly 50,000 men, women and children are dying every day from infectious diseases with antibiotic resistance as leading cause of death (who.int/whr/1996/media_centre/press_release/en/). Specifically, lower respiratory tract infection alone is a fourth leading cause of total annual deaths occurring globally from the diseases. A similar comprehensive analysis by Center for Disease Control and Prevention, USA stated that approximately 23,000 out of 2 million people suffering from antibiotic-resistant infections die every year and the prevention and treatment of microbial resistance imposes 20 billion USD additional burden on hospital expenses in USA (https://www.cdc.gov/drugresistance/about.html). Another report mentioned more than 33,000 deaths across the Europe in the year 2016 due to the resistance against antibiotics (https://ecdc.europa.eu/en/news-events/33000-people-die-every-year-due-infections-antibiotic-resistant-bacteria). As per the report by Centre for Disease Dynamics and Policy, India, nearly 50,000 children are dying every year in India due to Antimicrobial resistance (AMR) (https://www.icmr.nic.in/sites/default/files/ICMR_NEWS_23feb_8march.pdf). It is clear from the facts by different sources provided above that, antibiotic resistance is growing as a major challenge by not only affecting the millions of patients worldwide but also becoming a major burden on economy.

Developing the new antibiotics against the resistant mutant and improving the efficacy of the currently available antibiotics are the possible ways to fight with this major challenge. Unfortunately, developing the new antibiotics is a time consuming process associated with high risk of failure and simultaneously requires huge amount of money.

The effective approach would be to enhance the therapeutic efficacy of the existing antibiotics by the combination of adjuvants, reduction of enzymatic deactivation, prevention of efflux and facilitated penetration in microbial membrane and/or biofilm.

Antibiotic resistance may results due to mutation acquired from other species by genetic transfer, inappropriate prescription, inadequate diagnosis, improper therapy and even due to the physical modification in the living structure (R Benveniste and Davies 1973). Specifically, biofilm is a best example of antimicrobial resistance obtained due to physical modifications in microbes in response to the physiological stimuli such as pH, antibiotics, ions or oxygen (R Benveniste and Davies 1973).

Precisely, microbes have the propensity to adhere surface and start multiplication to generate micro colonies accompanied with secretion of extra polymeric substance (EPS). The EPS form the protective covering around the microbial colonies. These microbial colonies protected with EPS are commonly known as biofilm. It is a surface attached close aggregation of microbes encased in EPS, which exhibit high resistance against antibiotics and immune response. Consequentially, antibiotics fails to produce the sufficient concentration (minimum inhibitory concentration) needed in close vicinity of microbial colonies usually surrounded by EPS and requires the elevated level of antibiotics (up to 100 – 1000 times) to achieve the minimum inhibitory concentration, which may exaggerate the toxic effects (Del Pozo and Patel 2007, T Rybtke, O Jensen et al. 2011, Van Acker, Van Dijck et al. 2014, Pang, Raudonis et al. 2018).

The biofilm is comprised of alginate, other exopolysaccharides, proteins, nucleic acid, enzymes, ions and extracellular deoxyribose nucleic acid (eDNA) molecules, which are ultimately responsible for biofilm attachment, progression, and providing the three dimensional structure which ultimately makes the antimicrobial therapy ineffective or

less effective. The biofilm not only cause the poor cell penetration of the antimicrobials, but also responsible for inducing oxidative stress, overproduction of the efflux pump, ionic chelation of the charged antibiotics and the enzymatic degradation of drugs (Mayhall 2003, Wu, Moser et al. 2015). A biofilm can further induce the chronic disease condition, low grade persistent inflammation due to accumulation of polymorphic nucleocyte (immune mediator) and requires extra care for the treatment besides other clinical manifestation (Wu, Moser et al. 2015). Additionally, the biofilm is highly heterogeneous with different phenotypes depending on the physiological conditions, infection site and disease.

Pseudomonas aeruginosa, an omnipresent, opportunistic and highly heterogeneous gram negative bacterium with different phenotypes, is a major reason for most of the nosocomial infections including wounds and cystic fibrosis (CF) (Pang, Raudonis et al. 2018). *P. aeruginosa* is mostly associated with copious biofilm formation and this hinders the antibiotic therapy. Moreover, the ability to adopt different phenotypes makes the *P. aeruginosa* infection more resilient to the antibiotic therapy (Oliver, Cantón et al. 2000, Sauer, Camper et al. 2002, Kidd, Canton et al. 2018). Predominantly, extracellular DNA is a substantial structural constituent in the EPS of non-mucoid strain of *P. aeruginosa*. However, under certain specific pathophysiology like CF, *P. aeruginosa* invades the lung epithelia and shows the genetic transformation from non-alginate producing (nonmucoid) to alginate producing strain (mucoid) in presence of certain physical or biochemical stimuli such as pH, hypoxia, and temperature or may be the presence of the antibiotics. This transformation supports higher bacterial adherence in lung mucosa and promote immune escape (Sarkisova, Patrauchan et al. 2005, Tré-Hardy, Vanderbist et al. 2008, Alipour, Suntres et al. 2009, Horsman, Moore et al. 2012). Explicitly, extracellular DNA and alginate depending

upon the origin of the biofilm and disease, magnify the disease complication by facilitating cell to cell connectivity and cell to biofilm connectivity in conjunction with stimulating cell surface adhesion, cell accumulation, biofilm progression, biofilm establishment, inflexibility and providing the three dimensional structure. Simultaneously, being negatively charged, it averts the penetration of positively charged antibiotics (e.g. aminoglycosides) due to electrostatic interaction, thereby reduces the penetration and approach of the antibiotics to the bacteria (Whitchurch, Tolker-Nielsen et al. 2002, Chiang, Nilsson et al. 2013). Hence, the biofilm imposes the major hurdle to the antimicrobials. Conclusively, the presence of biofilm imposes the extremely high resistance to the antimicrobial therapy by hampering the drug diffusion in the biofilm, binding and repelling electrostatically to charged antibiotics and also helps in avoiding host innate immunity (Pozo and Patel 2007, Flemming and Wingender 2010, Brooks and Brooks 2014, Van Acker, Van Dijck et al. 2014, Wu, Moser et al. 2015).

Burns, covering ≥40% of total body surface area (TBSA) are classified as severe burns, which lead to 75% of total deaths due to biofilm associated septic wound infections (Church, Elsayed et al. 2006). Burn injury is a severe traumatic condition which damages the skin's frontline external protective barrier and provides open access to microbes *viz. S. aureus, E. coli*, and *P. aeruginosa* and requires the immediate hospitalization and critical care (Rafla and Tredget 2011). Additionally, biofilm occurrence in the wounds exaggerates the complication and hampers the wound healing.

Silver Sulfadiazine (SSD) is a non-ionized, water-insoluble, fluffy pale yellow color powder with poor permeability across the skin. SSD is considered as gold standard and is a most preferred drug for the treatment of burn wound infections (White and Cooper

2005). Though SSD efficiently acts against burn wound infections, yet burning sensation and delayed wound healing, observed due to fibroblasts and keratinocytes toxicity, are the major limitations linked with SSD therapy (Hidalgo and Domınguez 1998, Poon and Burd 2004). Moreover, the SSD therapy is ineffective against the biofilm protected wound infections.

Although, variety of nanoformulations based approaches, *viz.* aloe vera gel containing SSD nanosuspension (Barkat, Ahmad et al. 2017) and SSD loaded silk fibroin nanofibers (Jeong, Kim et al. 2014), evidently reduced the fibroblast toxicity and accelerated the wound healing yet, these are not effective against biofilm mediated infections. Hence a much more specific mechanistic approach is needed to eliminate the biofilm infections in burn wounds.

Secondly, CF is a life threatening disease caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene responsible for membrane transport of anions, which leads to multi-organ impairment and body fluid retention. Mutation to CFTR gene chiefly affects the lungs, which brings about compromised mucociliary clearance, thereby increases mucus retention, susceptibility for persistent microbial infection, and exaggerates pulmonary inflammatory response and cause progressive airways obstruction leading to complete respiratory collapse. Moreover, it may also cause exocrine pancreatic insufficiency, biliary disease and metabolic disorder (Accurso, Sontag et al. 2005, Flume, Mogayzel Jr et al. 2010). Among all the bacterium, *P. aeruginosa* is an opportunistic pathogen which affects the majority of CF patients leading to the severe complications and chronic infectious condition by developing the biofilm colonies (Hurley, Cámara et al. 2012, Owlia, Nosrati et al. 2014).

Apart from biofilm inflated therapeutic limitation, lungs further inflicts limitation for pulmonary drug delivery such as fast macrophage clearance, mucociliary clearance (though slow in cystic fibrosis) and lungs microenvironment created by lungs surfactants (including phospholipids, phosphatidylserine, DPPC, cholesterol, etc.) (Liang, Ni et al. 2015, Newman 2017). Pulmonary surfactants play a fundamental role in normal lungs functioning during gaseous exchange at lung air-liquid interface by reducing the surface tension. Any dysfunction to the lungs surfactant by inactivation or change in composition may adversely affect the alveoli and lungs efficiency, which may exhibit severe lungs disease including acute respiratory distress syndrome (Hidalgo, Cruz et al. 2015). Consequentially, the use of synthetic and external surfactant may adversely affect the lungs microenvironment and can cause severe damage to alveoli epithelium. Therefore, a specialized pulmonary delivery system is needed to eradicate the biofilm mediated *P. aeruginosa* infection in cystic fibrosis, which not only eliminates the associated biofilm microbes but also preserve the lungs microenvironments for improved pulmonary functions.

Contemporarily, several approaches, either to inhibit biofilm formation or for improving the antibiotic efficacy such as use of quorum sensing quenching agents (RNAIII-inhibiting peptide); biofilm dispersal by modifying the c-di-GMP (cyclic diguanylate monophosphate) target; bacterial amyloid inhibition; nanoparticles (polymeric or lipid), lectin inhibitor, iron chelation, biofilm dispersing agents, polymer with the intrinsic anti-biofilm property, phase therapy, electrochemical scaffold, nanoparticles and vaccine strategy have been investigated concurrently with antibiotic to actively eliminate the biofilm (Pozo and Patel 2007, Brooks and Brooks 2014, Wu, Moser et al. 2015).

Among the different biofilm treatment approaches, use of the deoxyribonuclease-I (DNase-I) for non-mucoid microbial biofilm infection in wounds and alginate lyase for mucoid *P. aeruginosa* biofilm in cystic fibrosis have gained the considerable attention worldwide. DNase-I breaks the phosphodiester bonds next to the pyrimidine nucleotides in DNA strands leading to the dispersal of biofilm matrix. Interestingly, DNase-I has been reported to reduce the viscosity of sputum carrying microbial biofilm and proved to be clinically safe when administered in CF patients. Consequently, the recombinant human DNase administration along with medication improved the pulmonary function in CF patients (Shah, Bush et al. 1995). A study also reported that, DNase-I significantly improves the antimicrobial activity of the aminoglycosides against biofilm associated infections. Till now the systemic and lung's biofilm infections were targeted by DNase-I with or without drugs however, in one of the objective of the present work drug loaded nanoformulation was developed and its efficacy was tested in combination with DNase-I against the wound associated biofilm infections. Similarly, alginate lyase (AgLase) in combination with antibiotics like aminoglycosides and others has been tested to improve the bacterial sensitivity to antimicrobials in biofilm infections. AgLase has the capability to disperse the architecture of biofilm, thereby improve the antibiotics distribution and therapeutic efficacy by hydrolyzing the negatively charged alginate. Moreover, phagocytosis has been reported to increase due to the enhanced immune response with alginate reduction (Mai, McCormack et al. 1993, Hatch and Schiller 1998, Ramsey and Wozniak 2005, Alkawash, Soothill et al. 2006). Hence, the enzymatic degradation of alginate reduces the three-dimensional structure of the matrix and thus enhances the penetration of antibiotic and its susceptibility.

Besides the use of biofilm hydrolyzing enzymes, biodegradable polymeric and lipidic nanoparticles have fascinated the worldwide researchers due to inherent properties of high drug loading, controlled release over the time, biocompatibility, accessible design and surface modification property for active targeting (Müller, Radtke et al. 2002, Baroli 2010, Kong, Chen et al. 2010, Wang, Zeng et al. 2011, Nagpal, Singh et al. 2013, Agrawal, Singh et al. 2017).

Nanoparticles facilitate the penetration into the biofilm and promote the close interaction of drug with microbial colonies (Khalid and El-Sawy 2017). Nanoparticles attach/fuse with cell wall/cell membrane of the microbes and deliver the drug in close proximity for longer duration, in that way prevent the microbial tolerance and prolong the inhibitory activity. Apart from the above advantages, nanoparticles provide the higher surface area to attain the physical and chemical modifications with various components (proteins, peptides, targeting ligand and enzymes etc.) for active targeting to the diseased site. The biofilm penetration ability of the nanoparticles can be further enhanced by functionalization of nanoparticles with biofilm hydrolyzing enzyme or co-administration of enzyme with nanoparticles (Toti, Guru et al. 2011, Xie, Zhu et al. 2011, Baelo, Levato et al. 2015).

In view of the above discussion, two different formulation strategies *viz.* lipidic and polymeric nanoparticles in combination with two different enzymes were developed and tested for the efficacy against the biofilm in burn wounds and CF.